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AN ANALYSIS OF ADENOVIRUS-INDUCED CRYSTALLINE INCLUSIONS

by

(C) ERIC BRUCE CARSTENS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "AN ANALYSIS OF ADENOVIRUS-INDUCED CRYSTALLINE INCLUSIONS" submitted by ERIC BRUCE CARSTENS in partial fulfilment of the requirements for the degree of Master of Science in Virology.

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To Ray and Halyna

'Namasta'

The truth is everywhere. Wherever you are, it's right where you are, when you can see it. And you can see it through whatever vehicle you are working with, you can free yourself from certain attachments that keep you from seeing it. The scientist doesn't stop being a scientist ... we never find out anything new, we just remember it.

Ram Dass

ABSTRACT

The intranuclear protein crystals formed in KB cells following infection with adenovirus type 5 were studied with respect to their morphological and biochemical characteristics.

Optical diffractometric and electron microscopic studies of these structures have enabled them to be assigned to the monoclinic crystal system in either the space group P2 or P2(1). The unit cell dimensions of the crystal were found to be a=50±2 nm, c=94±4 nm, beta = 95±1° and gamma = 90°.

Four methods were studied for their efficiency in selectively extracting the proteins comprising the crystals. These methods were: fixation and fracturing, suspension in 1% citric acid, suspension in 10% Tergitol and suspension in hypotonic buffer. The Tergitol-extracted proteins were analyzed by ion exchange chromatography, hydroxlapatite chromatography, gel filtration, and polyacrylamide electrophoresis. These studies revealed that large quantities of hexon, fiber, penton and core proteins were extracted from the nucleus. Analysis of the hypotonic buffer extracted proteins indicated that large quantities of hexon, fiber and core protein precursors were present in the intranuclear crystals. Imuno-gel diffusion experiments have shown that

the extracts reacted with both purified Ad5 antiserum and P-antigen antiserum, confirming the presence of virus-induced antigens in the extracts.

In vitro crystallization of proteins extracted from the infected cell nuclei resulted in the formation of structures similar in morphology to the *in vivo* crystals. The study indicated that the intranuclear protein crystals were composed of hexon, fiber and core protein precursor polypeptides.

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LIST OF ABREVIATIONS

Ad	adenovirus type
°C	degrees Celsius
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GuHCl	guanidinium chloride
ME	2-mercaptoethanol
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFU	plaque forming units
P&S	penicillin and streptomycin
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SST	sodium silicotungstate
TCID-50	50% tissue culture infectious dose
Tris	tris (hydroxymethyl) aminomethane
v/v	volume per volume
w/v	weight per volume
(1)	refers to crystal space groups, subscript 1

INTRODUCTION

In 1953, Rowe et al. discovered and isolated a new infectious agent from tissue cultures of human adenoids. In the following year, Hilleman and Werner (1954) isolated a similar agent from military recruits suffering from a respiratory infection. The agent was recognized as a virus, and in 1956, the group was assigned the name adenovirus.

Adenoviruses are mainly known to cause acute respiratory infections with accompanying cough, headache and fever, pharyngitis, and keratoconjunctivitis. However, enteritis, rashes, lymph node involvement, intussusception and myocarditis have been reported (cf. Wadell, 1970). Many adenoviruses have an oncogenic potential in newborn hamsters (Trentin et al., 1962) and have transformed cells (with reference to a change in cellular growth properties), including human cells, in vitro (Black, 1968). So far, 32 human, 23 simian, 6 bovine, 4 porcine, 2 canine, 1 murine, and 8 avian types have been isolated and characterized (cf. Wadell, 1970). The serotypes can be determined with type specific antisera which will either neutralize the infectivity of the homologous serotype, or inhibit the hemagglutination caused by the adenoviruses. Table I summarizes some of the properties of serotypes of human adenoviruses. It also indicates an apparent relationship between the hemagglutination subgroups, the fiber lengths

TABLE I[1]

Serotype	Hemagglutination Subgroup[2]	Oncogenic Subgroup[3]	Fiber Length	Crystal Spacing[4]
1.	III	C	28–31 nm	
2,5,6	III	C	28–31	60–70 nm
3,11	I	B	9–11	
4	III	D	17–18	25
7,14,16,21	I	B	N.D.	
8	II	D	N.D.	
9	II	D	12–13	
10	II	D	N.D.	
12	III	A	28–31	38
13,15,17,19 22,23,24,26 27,29,30	II	D	N.D.	
18,31	III	A	N.D.	40
20,25,28	I	D	N.D.	

1. Modified from Norrby (1969 b)
2. Rosen (1960): I, complete agglutination of monkey erythrocytes; II, complete agglutination of rat erythrocytes; III, incomplete agglutination of rat erythrocytes.
3. Schlesinger, (1969): A. high, cause tumors in hamsters within 2 months; B. weak, cause tumors in some animals in 4–18 months; C, nononcogenic; D, undesignated.
4. Weber and Liao (1969)

and the intertubule spacing of the adenovirus induced protein crystals.

The genetic information of the adenoviruses is contained in a linear, double stranded DNA molecule which has a molecular weight of 20-25x10^6 daltons (Pina and Green, 1965; Green et al., 1967; van der Eb et al., 1969). The DNA has been reported to be neither circularly permuted nor terminally redundant (Green et al., 1967), but recently, it has been shown to contain an inverted terminal complementarity (Garon et al., 1972; Wolfson and Dressler, 1972).

The typical design of the adenovirus particle, based on a study of electron micrographs of adenovirus type 5 (Ad5), was outlined by Horne et al. (1959). Detailed studies of the architecture of the virion of Ad5 (Valentine and Pereira, 1965) and of Ad3 (Norrby, 1966) have been presented. The capsid, consisting of 252 capsomeres, has an icosahedral shape with 5-3-2 symmetry, and a diameter of 70-80 nm. Twelve of the capsomeres, at the vertices of the icosahedron, are unique in having five nearest neighbours and are referred to as pentons (Ginsberg et al., 1966). Each penton consists of a penton base capsomere and a projecting fiber terminating in a small knob (Valentine and Pereira, 1965). The five surrounding capsomeres are called peripentonal hexons (Laver et al., 1968) due to their uniqueness of having the penton base as one of their six

nearest neighbours. The remaining 180 capsomeres (hexons) consist of 20 interlocking groups of nine hexon nonamers (Smith et al., 1965; Pereira and Wrigley, 1974). These capsid components account for approximately 60% of the protein content of the virion.

The structural proteins of the adenovirus virion correspond morphologically and immunologically with the large quantities of virus-specific proteins found free in cell extracts (Norrby, 1966). These proteins are soluble in the native state and are therefore referred to as soluble antigens. They are synthesized in the cytoplasm and are then transported to the nucleus where virions are assembled (Thomas and Green, 1966; Velicer and Ginsberg, 1968) . The structural proteins can be detected 2-4 hours before the maturation of virions (Russell et al., 1967a; cf. Schlesinger, 1969). When measuring antigen production by complement fixation 5-24 hours after infection under one step growth conditions, Philipson and Pettersson (1973) were able to show that the stuctural proteins are produced in the order hexon, fiber, penton base, and major core protein. It appears that all proteins synthesized in the cells at late stages of infection are coded by viral DNA (Ginsberg et al., 1967). A large excess of the structural proteins accumulates in the infected cell, since only approximately 5% of the total viral proteins appear in the mature virus particles (Bello and Ginsberg, 1969).

The excess pool of soluble antigens in infected cells has been isolated, purified, and characterized by numerous workers using techniques such as immuno-electrophoresis (Pereira et al., 1959) and ion-exchange chromatography (Klemperer and Pereira, 1959; Philipson, 1960; Wilcox and Ginsberg, 1961). Recently, Boulanger and Puvion (1973) have suggested a three step purification procedure consisting of neutral salt precipitation, ion-exchange chromatography, and adsorption chromatography, for large scale purification of hexon, fiber, and penton antigens.

Pettersson et al. (1967) described the electron microscopical appearance of the hexon as a complex structure with a diameter of 8-10 nm and a central hole 2.5 nm in diameter. Upon disruption of intact virions, hexons are released as aggregates of 9 capsomeres originating from the faces of the icosahedron (Smith et al., 1965; Laver et al., 1968; Prage et al., 1970). These aggregates have recently been called hexon nonamers (Pereira and Wrigley, 1974). Various methods including ultracentrifugation, sedimentation analysis combined with exclusion chromatography and x-ray crystallography have been used to determine the molecular weight of the hexon. The molecular weight appears to be in the range of 330,000-360,000 daltons (Franklin et al., 1971). The polypeptide composition of hexons was studied by Maizel et al. (1968) using a neutral, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis system. They found

that hexons contain a single component polypeptide of 120,000 daltons, implying that the hexon consists of 3 identical subunits. Hexons were first crystallized by Pereira et al. (1968). Various x-ray crystallographic studies have been performed (MacIntyre et al., 1969; Cornick et al., 1971; Franklin et al., 1971) These suggest that hexons preferentially crystallize in the space group P2(1)3, and that they contain several subunits in multiples of 3.

The penton base has been purified by techniques similar to those used for hexons. Due to its fragility and low concentrations in infected cells, the penton base is not as well characterized as the hexon. Morphologically, the penton base appears to be very similar to the hexon with a diameter of 8 nm, sometimes exhibiting a pentagonal outline, and a central hole 2.5 nm in diameter (Pettersson and Hoeglund, 1969). Aggregates of pentons (penton base + fiber) can exist as oligomers such as dimers, which act as complete hemagglutinins, and dodecons, which are symmetrically arranged groups of 12 (cf. Wadell, 1970). Neutral-SDS polyacrylamide gel electrophoresis (Maizel et al., 1968) indicates that the penton base is composed of a single polypeptide with a molecular weight of 70,000. Analytical centrifugation and sedimentation analysis indicate a molecular weight of 400,000-500,000 daltons for the penton base plus fiber complex. The penton base is sensitive to trypsin, being degraded to low molecular weight products at

concentrations of this proteolytic enzyme above 0.025 mg/ml.

Each penton base has attached to it a projecting fiber terminating in a small knob 4–6 nm in diameter. These fibers can be purified using similar techniques to those used for purification of the other capsid components. Although the diameter of the fiber is constant at 2 nm, the length varies greatly within the different subgroups of adenovirus – from 8-10 nm in Subgroup I through 12-17 nm in Subgroup II to 23–31 nm in Subgroup III (Norrby, 1969). Fibers from different subgroups can thus be separated by exclusion chromatography and under purified conditions, have been shown to crystallize (Mautner and Pereira, 1971).

The estimation of the molecular weights of Ad2 fibers as 200,000 (Philipson and Pettersson, 1973) and of fiber polypeptides as 62,000 in SDS gels (Maizel et al., 1968) suggests that the fibers contain 2-3 identical subunits. The functional and structural significance of the terminal knob is not known.

The capsid components of adenoviruses account for approximately 60% of the viral proteins present in the virion (Valentine and Pereira, 1965; Russell et al., 1967). A comparison of the amino acid composition of the virion including a concentration of 7.9 moles percent of the basic amino acid arginine versus the various capsid components (hexon, present in the largest quantity in the virion,

contains 4.7 moles percent of arginine) suggests the presence of additional arginine-rich proteins possibly on the inside of the virus particle. Further evidence for an internal core protein(s) was forthcoming when Russell and Knight (1967) found additional previously undetected viral antigens associated with the virus core after aging virus preparations. These new antigens, which they called the P-antigens, were produced in cell cultures early and late after infection with Ad5. The P-antigens can be detected by complement fixation using rabbit antiserum prepared against an extract of DNA-transcription inhibited (cytosine arabinoside) Ad5-infected cells. They reported that purified disrupted virus but not intact virions reacted with antisera directed against the P-antigen. This suggested that at least one of the components of the P-antigen is an internal protein of the virus. This was supported by Russell and Becker (1968) who found that the formation of a late component of the P-antigen was dependent upon a supply of exogenous arginine and that the major core protein was rich in arginine.

Basic viral core proteins, rich in arginine and antigenically different from the capsid were isolated from disrupted virions by acid extraction, (Prage et al., 1968, 1970; Boulanger et al., 1970), by acetone treatment (Laver et al., 1967; Russell et al., 1968) and by SDS treatment (Maizel et al., 1968). Laver (1970) isolated and described

an arginine-rich protein from Ad2. Prage and Pettersson (1971) isolated and purified what appears to be the same arginine-rich protein from Ad2 and Ad3 by acid extraction of disrupted virions followed by preparative polyacrylamide gel electrophoresis. They found that their major core protein had an arginine content of 21-23%, a high alanine content, and that this protein amounted to 70% of the soluble viral core proteins or about 14% of the total viral protein. The molecular weight was estimated to be 17,000 daltons. Everitt et al. (1973), using the SDS-disc electrophoresis system of Maizel (1971) studied the polypeptide pattern of Ad2 and determined that the core consisted of two polypeptides. One was the arginine-rich protein purified by Prage and Pettersson (1971), while the other was a polypeptide with an apparent molecular weight of 48,500. Electron microscopical examination of the core after heat, formamide, or acetone treatment of virions reveals a mesh-like, unorganized morphology; cores isolated by pyridine treatment of virions show a central dense area with extruding fibers (Philipson and Pettersson, 1973). Table II gives a summary of some of the polypeptides which have been indentified in the adenovirus particle.

Synthesis of adenovirus particles is strongly dependent upon the concentration of arginine in the growth medium (cf. Schlesinger, 1969). It has been postulated that

TABLE II[3]

Polypeptide[1]	Molecular Weight (daltons)	Corresponding Structural) Component
II	120,000[1]	Hexon
III	70,000[1]	Penton Base
IV	62,000[1]	Fiber
IVa	55,000[4]	Fiber-associated?
V	48,500[1]	Core
Va	36,000[4]	Precursor to VI
Vb	34,000[4]	Precursor to VIII
VI	24,000[2]	Hexon Associated
VIa	21,000[4]	Core precursor
VIb	20,000[4]	Core precursor
VII	18,500[2]	Major Core
VIII	13,000[2]	Hexon Associated
IX	12,000[2]	Hexon Associated
X	6,500[2]	Peripentonal Region?

1. Designation of Maizel et al., 1968 a, b.
2. From Everitt et al., 1973.
3. Modified from Philipson and Pettersson, 1973.
4. Designation of Ishibashi and Maizel, 1974.

an arginine-rich protein, the major core protein in particular, is a maturation factor (Rouse and Schlesinger, 1967; Russell and Becker, 1968). By adding an infected cell extract to arginine-depleted infected cells, Winters and Russell (1971) were able to obtain in vitro assembly of labelled, viral components into mature, infectious virus particles. However, Everitt et al. (1971) suggested that the majority of the proteins assembled into mature virus particles at arginine reversion (medium containing arginine was added to arginine-depleted infected cells) were formed after the addition of excess arginine and that the pool of excess structural units present during arginine depletion was not available for virus assembly (Rouse and Schlesinger, 1972). This would indicate that the latter proteins were present in a form which is not suitable for assembly into virions.

Morgan et al. (1957) were the first to recognize "extraordinary crystals . . . not composed of viral particles" in thin sections of Ad5-infected HeLa cells. They found the crystals were not stained by Feulgen reagents, indicating a lack of nucleic acid, but that they were stained positively using dyes specific for protein. Prior to this, the crystalline arrays seen in the cell nucleus had been thought to be composed only of whole virus particles.

Morgan et al. (1960) studied HeLa and HEp-2 cell cultures infected with Ad5 in both the light and electron

microscopes. They reaffirmed that the crystals consisted of protein and due to the absence of fluorochrome-stainable antigen, suggested that the crystals represented accumulations of non-viral proteins. A study of the fine structural changes caused by the replication cycle of Ad12 in KB cells (Martinez-Palomo et al., 1967) revealed the formation of four types of nuclear inclusions plus "peculiar striated protein structures". They described the various inclusions as follows:

(a) Type I inclusions: dense spherical inclusions which were shown to fix antibodies against viral structural antigens (Levinthal et al., 1967) using an indirect immunoferritin technique.

(b) Type II inclusions: irregular, moderately dense inclusions formed by accumulations of fibrils. They appear to be depositions of protein associated with nuclear or viral DNA.

(c) Type III inclusions: round accumulations which enlarge progressively during the viral replication cycle until they occupy most of the nucleus. They may consist of viral DNA.

(d) Type IV inclusions: dense reticular inclusions, resistant to proteolytic enzymes and which fix ferritin-labelled antibody directed against viral structural proteins. They may be considered as a mixture of structural

antigen and viral DNA.

(e) Striated protein structures: cross-striated lamellae consisting of parallel depositions of two thin filaments, 5 nm thick, between thick filaments, 6 nm thick, with a periodicity of 38 nm between thick filaments. The authors state that these structures are different from the large protein crystals seen in other adenovirus infections.

Other types of adenoviruses were studied with respect to their ability to stimulate crystal formation in infected cells. Weber and Liao (1969) studied Ad2, Ad4, Ad6, and Ad18 and the crystals they induced. They concluded that on the basis of fine structure, crystals induced by Ad2 and Ad4 were homologous to those induced by Ad5. The highly oncogenic viruses, Ad12 and Ad18, seemed to induce identical crystal formations, but these were different from the others studied. It appears that crystal formations induced by common subgroup members have very similar characteristics.

A model of the crystal structure, based on the results of two different extraction procedures was proposed by Boulanger et al., (1970). They found that by treating cells infected with Ad5 with either 1%(w/v) citric acid or 0.15%(v/v) Cemusol (a non-ionic detergent), the crystals were selectively extracted from the nucleus of the cells. They also reported the presence of adenovirus structural proteins in the supernatant of this extract. When they used

antisera prepared against hexons to study the indirect immunofluorescent staining properties, they observed large fluorescent polyhedral intranuclear inclusions, corresponding to the crystals. They thus proposed that the crystals were accumulations of either excess viral material or a side product of virus infection.

To further elucidate the nature of the crystals formed in Ad2 infected cells, Henry and Atchison (1971) used direct immunofluorescent staining with fluorescein isothiocyanate-labelled Ad2 antibody (prepared against disrupted Ad2 virions) to demonstrate high intensities of staining in the crystal structure. When they studied the crystals using an indirect staining method with hexon and fiber antiserum, they found very little fluorescence in the crystal. This suggested that the crystals were composed of internal virus components. In fact, they were able to show positive fluorescence of the crystals with phenanthrenequinone, a compound which forms a fluorescent condensate with arginine (Russell et al., 1971). Interferon was used to pretreat the cell cultures, thereby causing a reduction in crystal formation. As interferon has little effect on the host cell's protein synthesis, this reduction in crystal formation was seen as a result of inhibition of virus protein synthesis.

In 1972, Marusyk et al. (1972) reported that the crystals did not fluorescent stain with highly specific

antisera prepared against hexon, fiber or penton, but rather, that the crystals did fluoresce in the presence of antisera directed against the Ad5 P-antigen and the Ad2 arginon. Both of these antigens are related to adenovirus core proteins (cf. Marusyk et al., 1972). They also reported that by incubating infected cells in the presence of a very low concentration of arginine, blockage of virus maturation occurred but crystals were still formed, indicating that formation of the protein crystal may require less free energy than assembly of the virion; the latter requiring some other arginine-dependent maturation protein.

Wills et al. (1973) studied cells infected with temperature-sensitive mutants of Ad5 with respect to crystal formation. They used temperature-sensitive mutants isolated by Williams et al. (1971) and characterized into complementation groups by Russell et al. (1972). They reported that crystal formation did not occur when either capsid or 'late' antigens were not formed. Their work also implied that fiber antigen but not hexon antigen expression was related to crystal formation. They were also able to show that crystal morphology was temperature dependent, i.e. in many cases, crystal subunits were arranged in a rather irregular pattern at 33°C with both the wild type strain and the temperature-sensitive mutants.

Very recently, Lifchitz (personal communication) has constructed a three dimensional model of the protein

crystal, based on crystallographic data. His model consists of helical polyhedral tubules composed of hexons collapsed onto a tubular fiber of basic core protein.

The present study was undertaken to investigate further the morphological and biochemical nature of the adenovirus type 5-induced intranuclear protein crystals and to determine more definitely the relationship of these crystal proteins to viral specific proteins.

MATERIALS and METHODS

1. Cell Cultures

KB cells (CCL17; derived from a human oral carcinoma) were normally maintained in Blake bottles. After 75-90% monolayers were formed, the medium was removed and the cell sheet washed with 10 ml of 0.25% (w/v) trypsin. Most of the trypsin was then removed and the bottles were incubated at 37°C for a sufficient time to detach the cells from the glass. The cells were then removed from the bottles with a small volume of fresh growth medium and diluted to the desired volume with fresh growth medium so that each Blake bottle to be seeded received an aliquot of 100 ml of cell suspension (about $5x10^5$ cells/ml). A monolayer of 75-90% confluency was usually formed in 1-2 days, and at that time, they were: a) infected with virus, b) used as control cultures, or c) incubated at 37°C until conditions required re-seeding (passaging).

2. Cell Culture Medium

Cell cultures were seeded in minimal essential medium-Earle's base (MEM-Eagle's Modified Auto-POW, Flow Lab.). This powdered medium was dissolved in distilled water and autoclaved for 15 minutes at 15 psi pressure, 120°C. Prior to use, sodium bicarbonate and glutamine were added to final concentrations of 0.1% (w/v) and 0.002 M respectively.

Five percent calf serum was added for growth medium and 2% calf serum was added for maintenance medium. To each liter of medium, 10 ml of an antibiotic solution (100,000 units of penicillin, 100,000 ug of Streptomycin per ml,. (P&S), GIBCO) were added prior to use.

Amino acid-free Eagle's medium was made up as follows: 0.1 M sodium chloride, 5.1 mM potassium chloride, 0.86 mM magnesium chloride, 5.3 mM glucose, 3.4 mM calcium chloride, 4 mM glutamine, 0.04% phenol red, 7.5% sodium bicarbonate as required, 10 ml/liter penicillin-streptomycin stock solution, 10 ml/liter BME vitamin solution (100 times concentrated, GIBCO). This stock solution of medium was made up to 1/10 concentration of amino acids by adding 1 ml/liter of 100 times concentration amino acids (GIBCO).

3. Virus Production and Purification

Ten Blake bottles of KB cells at approximately 75% confluency were routinely used for propagation of Ad5. Prior to infection of the monolayer, the cell culture medium was removed, and 20 ml of a virus suspension (10 PFU/cell) in phosphate buffered saline (PBS) were added to each of the cell monolayers. The infected monolayers were then incubated at 37°C for 60 min with occassional swirling of the suspension over the monolayer. After the 60 min adsorption period, the virus-containing PBS was removed and 60 ml of fresh cell culture maintenance medium was added. The

infected monolayers were then incubated at 37°C for a further 48-72 hours. At that time, when complete cytopathic effect was usually evident, the medium containing the infected cells (at these late times of infection, almost all the cells were floating free in the medium) from all the infected Blake bottles was pooled and centrifuged at 800 g for 15 min. The cell pellet was washed three times with PBS and once with 0.02 M Tris-HCl, pH 9. The pellet was then suspended in 30 ml of 0.2% (w/v) sodium deoxycholate in 0.02 M Tris-HCl, pH 9, and gently mixed at room temperature for at least 60 min. Cell debris was removed by centrifugation at 800 g and the supernatant was layered on top of a pre-formed CsCl gradient consisting of 5 ml CsCl, buoyant density 1.39 g/cc, 5 ml CsCl, buoyant density 1.32 g/cc, and 5 ml CsCl, buoyant density 1.20 g/cc. The step gradient was centrifuged at 42,000 g in a Spinco SW 25.1 rotor for 2 hours (Marusyk et al., 1972). After this centrifugation, a definite opalescent band, corresponding to Ad5 virions, was easily removed from the centrifuge tube with a Pasteur pipette. This virus suspension was further purified by mixing it with a small volume of CsCl, buoyant density 1.34 g/cc, and centrifuging the suspension at 100,000 g in a Spinco SW 39 rotor. In this way, a high concentration of virions in CsCl was obtained.

4. Virus Assay

Two methods of assaying for viral infectivity were utilized:

a) Plaque assays for Ad5 infectivity were carried out using HeLa cells ($1x10^6$ cells/ml) seeded onto disposible plastic tissue culture dishes (60 mm diameter, Falcon) and maintained in MEM tissue culture medium supplemented with 5% calf serum. Serial ten-fold dilutions of Ad5 were prepared in PBS, and from each dilution two plates were inoculated with 0.5 ml each. The plates were rotated gently at 10 min intervals throughout the adsorption period of 60 min at 37°C. After adsorption, the cell monolayer was covered with melted agar-medium suspension prepared in the following manner: A 60 ml volume of 1.8% Noble agar (Difco) in distilled water was melted and cooled to 43°C in a water bath. Sixty ml of solution containing 25.5 ml distilled water, 12 ml of fetal calf serum, 2.4 ml of 100 times concentrated P & S, 1.5 ml of 7.5% (w/v) sodium bicarbonate, 1.2 ml of 100 times concentrated vitamins and 3 ml of 1 M HEPES (pH 7.4) were added to the melted agar and mixed. Six ml of this overlay medium was added to each infected and control plate. After the overlay medium had solidified, the plates were inverted and incubated at 37°C. Two ml of fresh overlay medium were added in a similar fashion every

2 days. On the eighth day, 3 ml of overlay medium containing 0.0002% neutral red were added to each plate. This was done in the absence of artificial lighting. The plates were incubated for 2 more days and then examined for produced plaques.

b) The 50% tissue culture infectious dose (TCID-50) of Ad5 was determined in tube cultures of HeLa cells maintained in MEM medium supplemented with 2% calf serum. Serial ten-fold dilutions of Ad5 were prepared in PBS and from each dilution, 4 tube cultures were inoculated with 0.1 ml each. After adsorption for 30 min at 37°C, fresh MEM medium containing 2% calf serum was added to each tube. Examination of the cell monolayers for CPE was made daily for 10 days after infection, and the log TCID-50 was calculated by the Reed and Muench (1938) method.

5. Micro Gel Diffusion

Micro gel diffusion plates were prepared by the technique of Krause and Raunio (1967). A plexiglass matrix (6 wells) containing 2.5 mm diameter steel balls in each well was placed on top of an acid-cleaned 5x5 cm glass slide and allowed to warm near a bunsen burner. Short pieces of fishing line (0.2 mm diameter) were placed under the corners of the matrix and hot 1% (w/v) agarose in PBS was added to

one side of the matrix and allowed to flow by capillary action between the matrix and the glass slide. A uniform weight (#5 rubber stopper) was placed on top of the matix and the agar was allowed to solidify. The fishing line was removed and the 4 edges were sealed with hot agarose. Prior to use, the steel balls and excess agarose were removed from the matrix wells. Samples (25 ul) were added and the gels were incubated for 72 hours at room temperature in a humidity chamber.

After incubation, the matrix was carefully removed and the agarose-covered slides were washed overnight in PBS. The slides were stained with amido black (0.1 g amido black, 50 ml 12% acetic acid, 50 ml 1.6% sodium acetate) for 30 min, then destained in 7% (v/v) acetic acid for 2-3 hours. After washing with PBS, the agarose was allowed to dry onto the slides overnight.

6. Electron Microscopy

Electron microscopical examination of all samples was performed on a Philips EM300 electron microscope operated with an 80 KV beam. Cell samples for observation in the electron microscope were fixed and embedded using the technique described by Doane et al. (1974). The cell samples were washed in PBS and then fixed in a freshly prepared solution of 1 part 2.5%(v/v) glutaraldehyde to 2 parts 1%(w/v) osmium tetroxide in Millonig phosphate buffer

(Millonig, 1962) for 30 min at 4°C. The fixed specimens were then dehydrated with acetone as follows: 70% acetone, 2 changes of 5 min each; absolute acetone, 3 changes of 5 min each. After 10 min in a 1:1 mixture of absolute acetone and Epon 812 embedding material (Fisher) and 10 min in 100% Epon, the specimens were transferred with a Pasteur pipette to the bottom of a BEEM (E. F. Fullum) capsule, covered with fresh Epon and heated at 85°C for 2 hours to achieve polymerization of the embedding material.

Thin sections of embedded cells were cut with a diamond knife on a Reichert OM U2 ultra-microtome and mounted on acid-washed, 3 mm copper grids (400 mesh, E. F. Fullam). The sections were stained in 5% (w/v) uranyl acetate in methanol for 10 min and lead citrate (Reynolds, 1963) for 2 min. Electron micrographs of tilted specimens were obtained on a goniometer stage mounted on the Philips EM300.

Specimens requiring negative staining were placed on formvar coated 400 mesh grids, previously covered with a thin layer of carbon. The appropriate stain was then applied and drained with a piece of filter paper. The specimens were examined immediately in the electron microscope.

7. Polyacrylamide Gel Electrophoresis (PAGE)

Acrylamide (Eastman 5521) and N-N'-methylene bis-acrylamide (Eastman 8383) were recrystallized using the

method of Loening (1967). Gels, 6mm in diameter and 90 mm in length, were formed in acid-cleaned glass tubes. The SDS-disc system of Maizel (1971) was employed, using a 3% spacer gel layered above a 13% resolving gel. The resolving gel was made up in Tris-HCl buffer, pH 8.9 (48 ml 1 M hydrochloric acid plus 36.3 g Tris/100 ml distilled water) containing 0.1% (w/v) sodium dodecyl sulfate (SDS, Fisher). Polymerization was induced by final concentrations of 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED, Eastman 8178) and 0.05% (w/v) ammonium persulfate (Baker). The spacer gel was formed in Tris-phosphate buffer, pH 6.7, (25.6 ml 1 M phosphoric acid plus 5.7 g Tris/100 ml distilled water) containing 0.1% SDS. Polymerization was induced by the addition of final concentrations of 0.05% (v/v) TEMED and 0.1% (w/v) ammonium persulfate.

The proteins used as standard molecular weight markers were; bovine serum albumin (68,000), fumerase (49,000), lactate dehydrogenase (36,000), trypsin (23,300), and myoglobin (17,200). Molecular weights as determined by Weber and Osborn (1969) were assumed. As the polypeptide composition of the Ad5 virion has been well characterized, and the molecular weights of the polypeptides have been determined, purified disrupted Ad5 virions were also used as marker proteins.

Proteins for analysis were solubilized by heating at 95°C for 5 min in 1% SDS, 10% glycerol, 0.1% 2-

mercaptoethanol, and 0.002% bromphenol blue in 1/10 concentration spacer gel buffer. Samples were routinely applied in a 0.1 ml volume to the top of the spacer gel after the electrode buffer (6 g Tris plus 28.8 g glycine/1000 ml distilled water, 1% SDS, pH 8.4) had been poured over the gels. Electrophoresis was performed using 3 mA/gel for 3 hours after which they were removed from the glass tubes and stained for 3 hours in 0.2% (w/v) Coomassie Brilliant Blue R (Sigma) in 1:7:7 acetic acid:methanol:distilled water. Destaining was achieved either by diffusion or by electrophoresis in 7% acetic acid, 5% methanol in distilled water.

8. Radioactive Labelling

^{14}C-arginine (NEC-267, L-arginine-^{14}C, 313 mCu/mM) and ^{3}H-alanine (NET-130, DL-alanine-3-^{3}H, 30.6 Cu/mM) were obtained from New England Nuclear.

Four hours before infection with Ad5, the growth medium was removed from the KB cell monolayer. The cells were washed once and then covered with amino-acid free medium. At the time of infection, the medium was removed and Ad5 in PBS was added and allowed to adsorb for 60 min. The inoculum was removed and 1/10 concentration amino-acid medium was added. Six hours after infection, either ^{14}C-arginine and/or ^{3}H-alanine were added (at a concentration of 1 uC/ml ^{14}C-arginine and 5uC/ml ^{3}H-alanine). At 16 hours after

infection, the labelled medium was removed. The monolayer was washed once with normal maintenance medium, then covered with this medium and incubated at 37°C. The cells were harvested 48-60 hours after infection.

9. Radioactive Counting

Liquid samples were spotted on filter papers and allowed to dry. After placing into plastic scintillation vials, 5 ml of scintillation cocktail (3g 2,5-diphenyl-oxazole, (PPO), and 0.5 g p-Bis[2-(5-phenyloxazolyl)]-benzene, (POPOP), in one liter toluene) were added to each vial.

Slices from polyacrylamide gels approximately 0.9 mm thick were placed in plastic scintillation vials, covered with 0.6 ml of 3% (v/v) piperidine (Baker) and incubated at 56°C for 90 min. Five ml of scintillation cocktail (250 ml Triton X-114, 3 g PPO, 0.2 g POPOP, in one liter toluene) were added to each vial .

All samples were counted in a Beckman LS-250 scintillation counter.

10. Column Chromatography and Gel Filtration

a) Ion exchange columns 1.6 cm in diameter, were prepared with the appropriate buffered suspensions, packed by gravity to a height of 30 cm.

b) Gel filtration columns, 2.4 cm in diameter, were prepared with the appropriate buffered suspensions, and packed by gravity to a height of 80 cm. The hydrostatic pressure was maintained at 10-13 cm to prevent packing of the column.

11. Buffer Solutions

Buffer solutions required for this research project were prepared from tables listed by Gomori (1955).

11. Manuscript Production

This thesis was drafted, edited, and printed using the FMT program and the IBM 360/67 computer of Computing Services, University of Alberta.

EXPERIMENTAL PROCEDURES AND RESULTS

1. Electron Microscopy

Electron microscopic examination of Ad5-infected KB cells 48-60 hours after infection, revealed the presence of typical adenovirus-induced inclusion bodies including protein crystals (Plate 1.). Depending upon the sectioning angle, transverse, diagonal or longitudinal sections of the crystal were seen (Plate 2.). Longitudinal sections were seen more often than transverse sections. The crystals were generally 2-3 microns in width, but varied greatly in length up to approximately 10 microns.

At early stages of crystal formation, the mother liquor surrounding the crystals and the numerous virus particles appeared to be disorganized or uncrystallized protein material. At late stages of infection, the mother liquor was not seen, as virus and protein crystals almost totally occupied the nucleus.

Inspection of electron micrographs of transverse sections through the protein crystal revealed a crystalline-like array of faintly contrasted parallel tubules with very electron dense tubular centers. These electron dense centers seemed to be connected to neighbouring centers by thin filaments. The diameter of the whole tubule was found to be approximately 47 nm while the electron dense central tubule

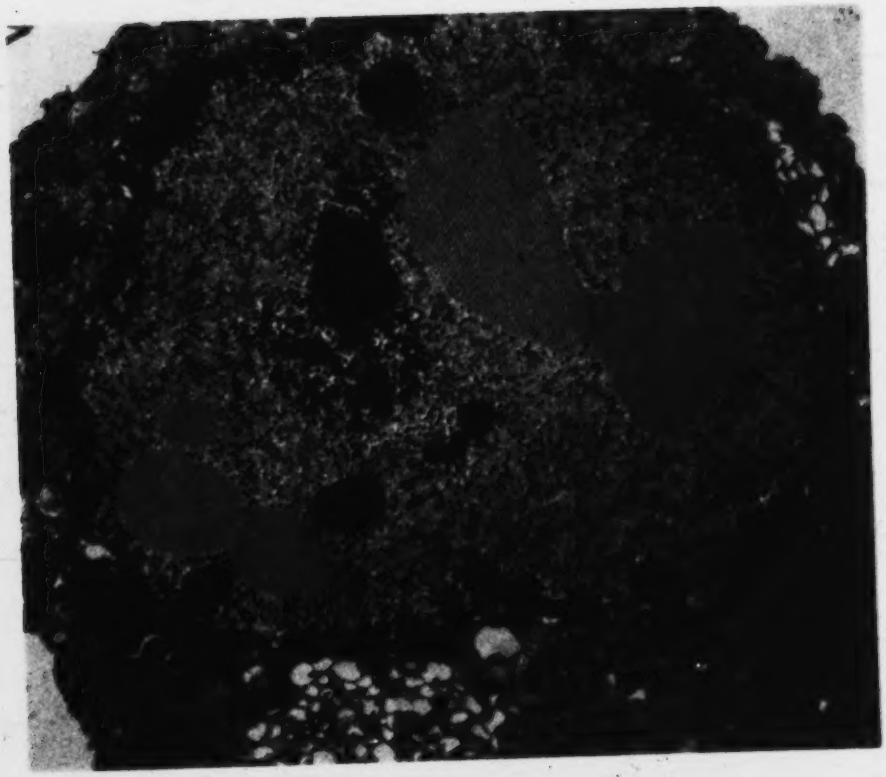

Plate 1.

Normal appearance of a KB cell nucleus, 48 hours after infection with Ad5. Note the margination of the nuclear chromatin and the presence of virus-induced intra-nuclear inclusion bodies, types I, II, III, IIV and several large protein crystals. X 11,200.

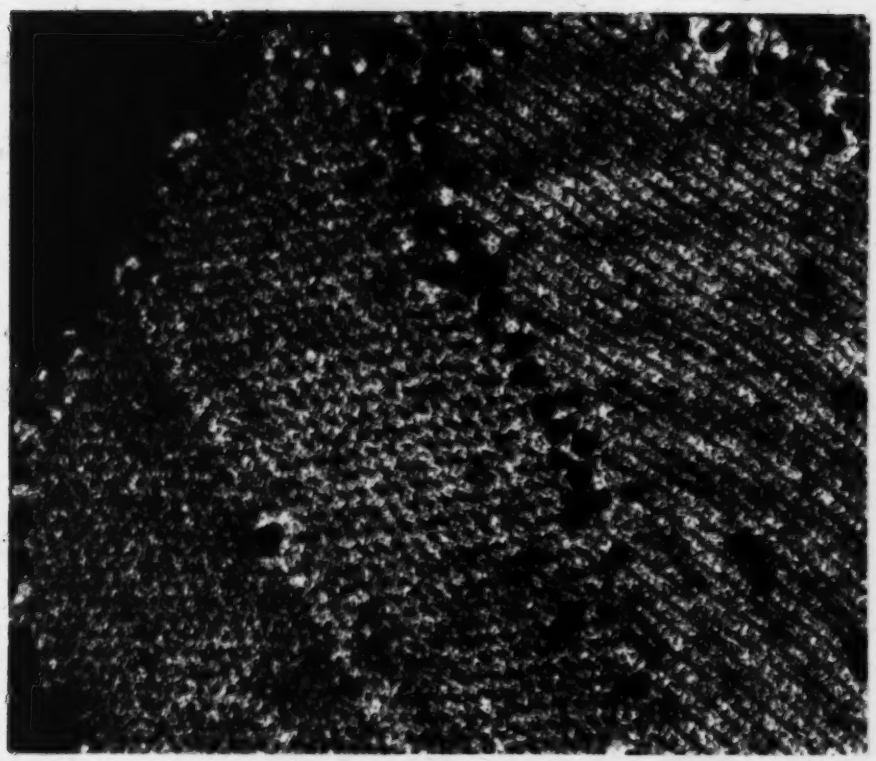

Plate 2.

High magnification micrograph of an infected KB cell nucleus showing three different orientations of the protein crystals. Note the close proximity of virus particles to the borders of the crystals and the similar staining densities of the virus capsids and the tubule walls in the longitudinal section. X 43,000.

had a diameter of about 22 nm. The crystals were usually six-sided in cross section, implying hexagonal symmetry of the subunit tubules. Longitudinal sections revealed long, parallel rows of filaments, arranged in doublets, 22 nm across. The inter-doublet spacings varied from virtually no spacing in some sections to 35 nm in others, depending upon the sectioning angle. Electron micrographs obtained after tilting the crystal in different directions relative to the electron beam revealed how different longitudinal arrangments could be formed, simply by observing different planes (Plates 3a, 3b, 3c and 3d).

By measuring the center-to-center inter-tubule distances in transverse sections, values of 50 nm or 55 nm were obtained, dependent upon in which particular direction the measurement was made (Plate 4.). This suggested that the two dimensional symmetry was not hexagonal, but either rectangular (two dimensional space group cm) or oblique (two dimensional space group p2; International Tables for X-ray Crystallography, 1952).

2. Optical Diffraction

Optical diffractometry studies were carried out in the laboratory of Dr D. Scraba (Department of Biochemistry, University of Alberta). These patterns were obtained by examining negatives of high magnification electron micrographs of transverse sections of the protein crystals

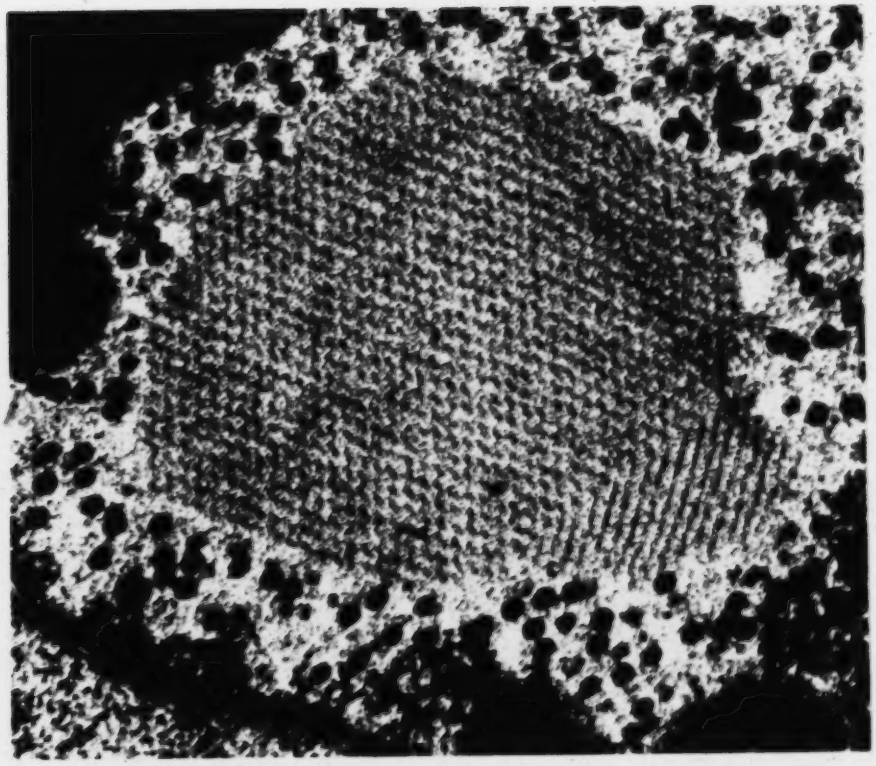

Plate 3a.

Ad5 induced protein crystal in a KB cell nucleus. Diagonal section, normal to the objective lens.(0^{o} tilt) X 54,000.

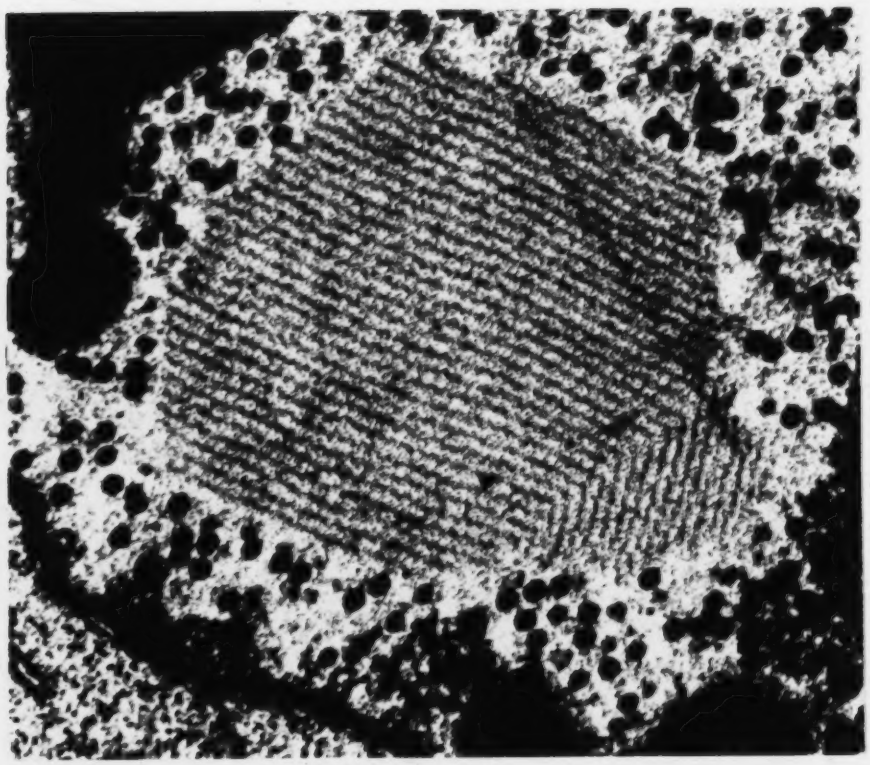

Plate 3b.

Same section as in Plate 3a., tilted 18°. Note the rows of parallel tubules which are now visible. X 54,000.

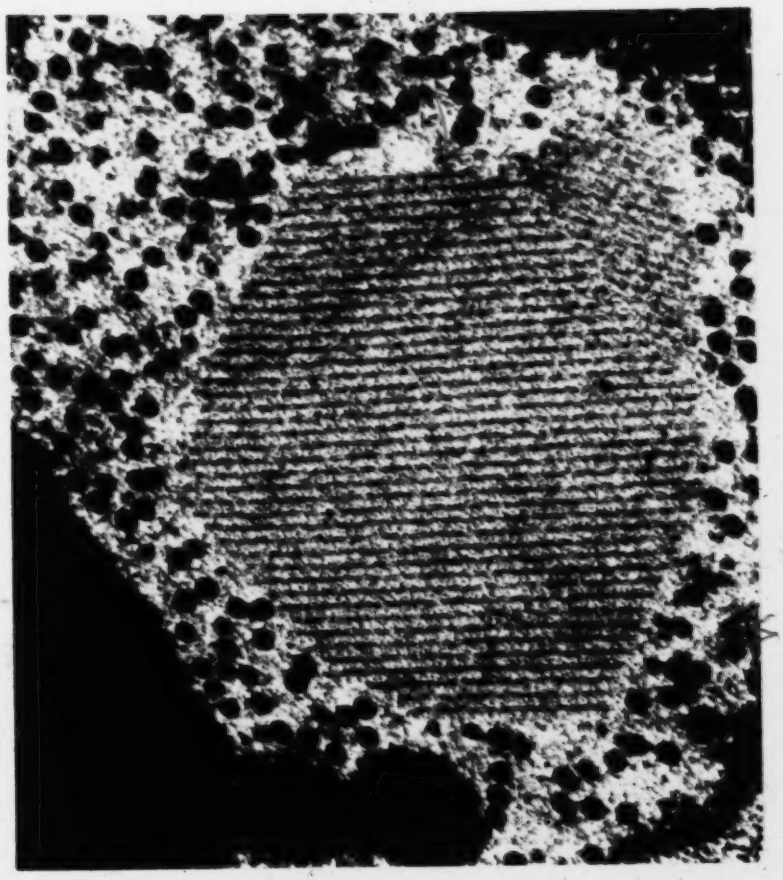

Plate 3c.

Same section as in Plate 3a., rotated 65° in the horizontal plane and then tilted 30°. A different set of rows of tubules from those in Plate 3b. are seen. X 54,000.

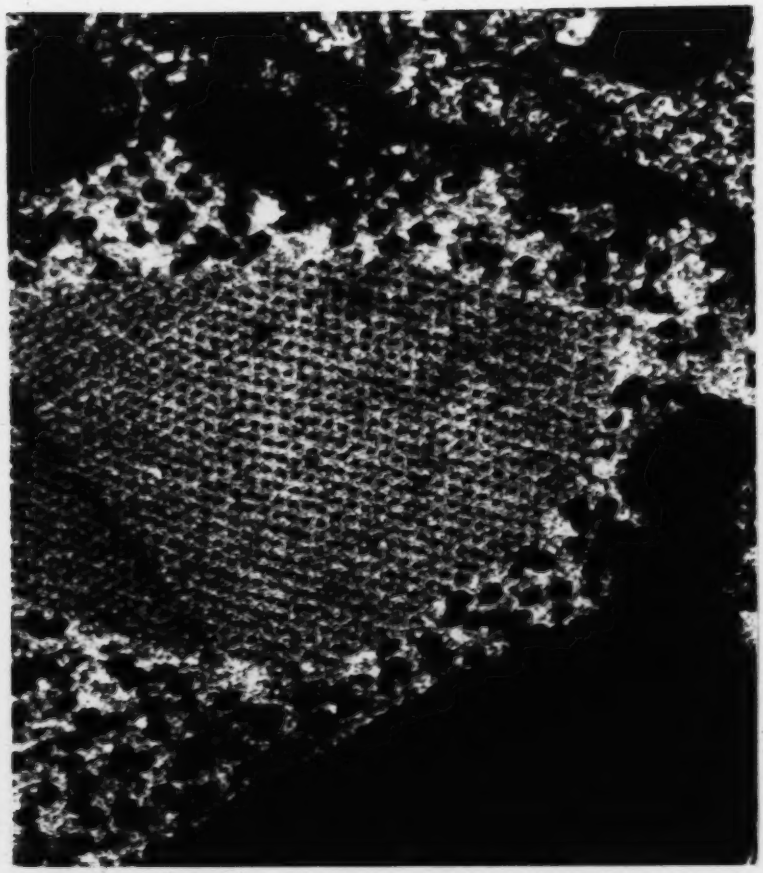

Plate 3d.

Same section as in Plate 3a., rotated approximately 180° in the horizontal plane and tilted 38°. Note that this micrograph shows that the diagonally cut tubules have been tilted towards the vertical axis (parallel to the electron beam) to reveal the crystal lattice whereas, in Plate 3b., the tubules have been tilted away from the vertical axis, revealing the rows of tubules. X 54,000.

q

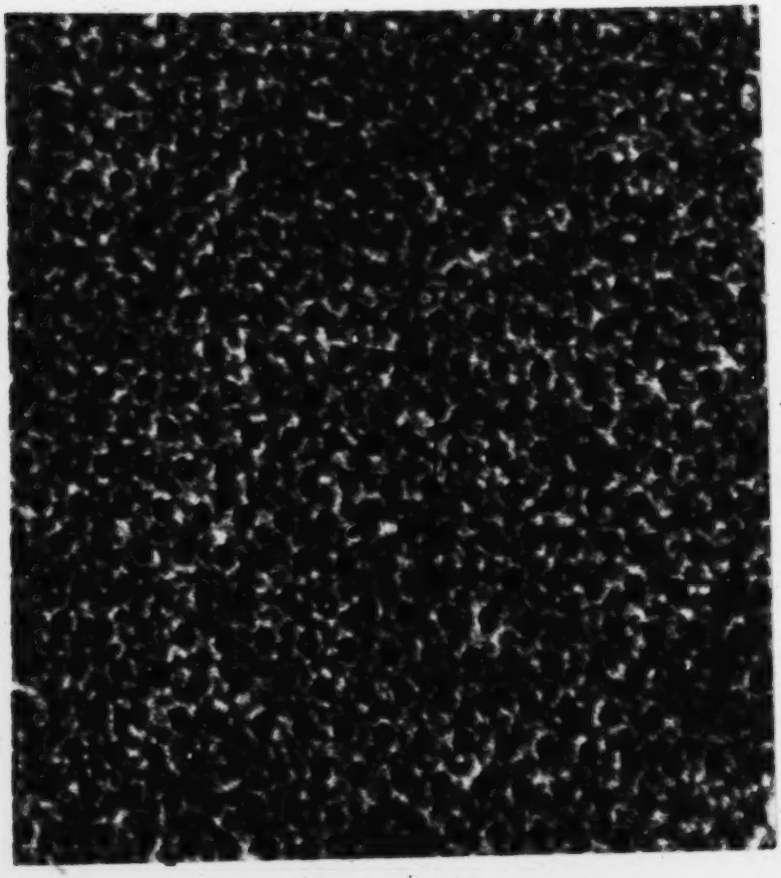

Plate 4.

High magnification micrograph of a transverse section, indicating the x and z axis. As most of the sections were very irregular in consistancy at these high magnifications, measurements were carried out on over 100 different sections to obtain the average values presented in this study. X 130.000.

in a Polaron optical bench (Dida Sciences Inc, Montreal) operated with a helium-neon light source (Model 8001, 2 mW uniphase output). The patterns so obtained (Plate 5.) were examined for symmetry elements. As shown in Fig 1., the angle formed between the two major axis designated X^1 and Z^1 was found to be 85°. As this is in reciprocal space, the corresponding angle in real space (i.e. the protein crystal) would be 95°. As shown in Fig 2., an angle of 95° can be formed between two major rows of tubules, designated X and Z. These results indicate that the two dimensional space group is p2, based on an oblique unit cell with an 'a' distance along the X axis of 50±2 nm and a 'c' distance along the Z axis of 94±4 nm. Angle beta was 95°±1° while angle gamma was 90°. Longitudinal sections were not examined in the diffractometer, thus the 'b' distance, the unit cell length along the Y axis was not determined.

3. Crystal Protein Extraction

Four different extraction procedures were investigated for their ability to selectively remove the proteins comprising the crystals from the infected cells. Attempts were made to isolate whole, intact, infected cell nuclei. However, it was observed that the cytoplasmic and nuclear membranes became very fragile after infection with Ad5, and that any disruption of these membranes resulted in the loss of the protein crystal inclusions (cf. Marusyk et al.,

Plate 5.

Optical diffraction pattern obtained following laser beam optical diffractometric analysis of thin sections of the protein crystal material.

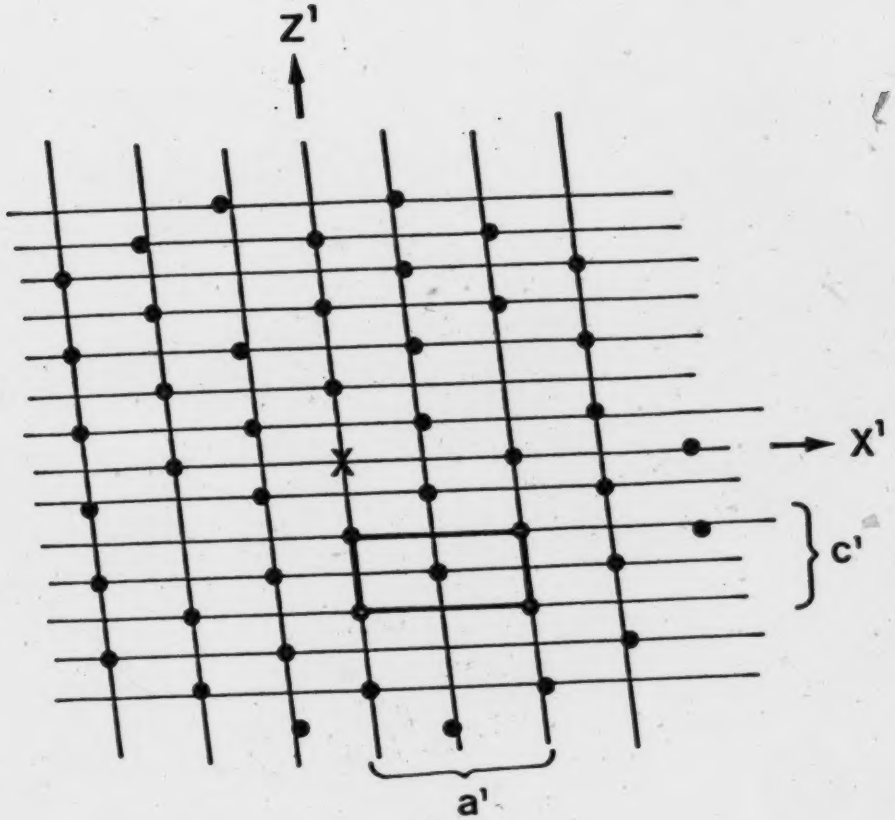

Figure 1.

Diagramatic representation of the diffraction pattern seen in Plate 5. The intensity spots were traced directly from the diffraction pattern and the reciprocal lattice lines were drawn. The unit cell in reciprocal space is indicated by a'c'. The Y' axis is perpendicular to the page.

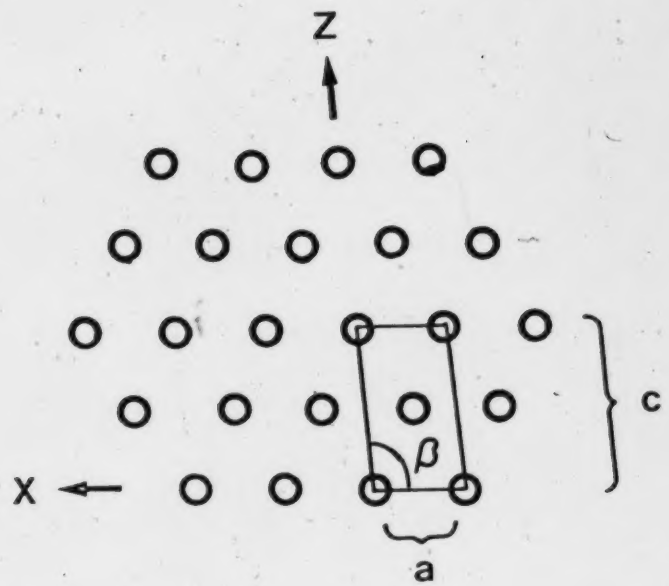

Figure 2.

Diagramatic representation of the two dimentional crystal lattice seen in transverse sections. The drawing was derived from information on electron micrographs and diffraction patterns. The circles represent the electron-dense tubules seen in transverse sections. a=50 ± 2nm, c=94 ± 4nm, beta=95 ± 1°.

1972). An extraction method which was fairly gentle, but selective was therefore required. In all cases, 10 Blake bottles of Ad5-infected KB cells were harvested 48-60 hours after infection. The cells were alternately centrifuged at 800 g for 10 minutes and washed in PBS three times before any procedure was initiated. The resulting cell pellet contained approximately 2 x 10^9 cells. Control uninfected cells, were subjected to the various extraction procedures and embedded for observation in the electron microscope. Control infected cells, not subjected to the extraction procedure, were examined in all cases for normal crystal formation.

a. Fixation and fracturing:

The cell pellet was suspended in 30 ml 0f 12.5% (v/v) hydroxyadipaldehyde (J. T. Baker Chemical Co.) in 0.1 M phosphate buffer, 0.44 M sucrose, pH 7.5 for 4 hours at 4°C (a mild fixative shown to allow retention of antigenic properties; Sabatini et al. 1963). The fixed cells were then subjected to either sonication for a total of 3 minutes with a Bronwill Biosonik III sonicator, or fracturing at 800 psi in a Sorvall Ribi Cell Fractionator. The cells were then centrifuged at 1200 g and embedded in Epon for thin sectioning. The thin sections of both the sonicated and the fractured cells showed broken cells and cell fragments. Protein crystals were

still embedded in the nucleus or were associated with cell remnants - none were seen as separate entities.

b. Citric acid extraction:

The cell pellet was suspended in 30 ml of 1% (w/v) citric acid in PBS. After mixing for 4 min, the suspension was centrifuged at 1200 g for 10 min, and the pellet was embedded in Epon for thin sectioning. When examined in the electron microscope, the thin sections revealed that all the cells had been completely disrupted, as only membranous material, chromatin and a few virus particles were seen in the cell remnants (Plate 6.).

c. Tergitol extraction:

Tergitol NPX (1:10.5 nonylphenol-ethylene oxide condensate, J. T. Baker Chemical Co.), a non-ionic detergent, was used at a concentration 10% (v/v) in PBS. Thirty ml were added to the cell pellet and the suspension was agitated on a Vortex mixer for 5 min. The cells were centrifuged at 1200 g for 10 min and embedded in Epon. Holes were visible in the nucleus, corresponding in size and shape to the protein crystals, and at times contained traces of the crystal structures (Plate 7.). Very few recognizable structures were seen in the cytoplasm, which along

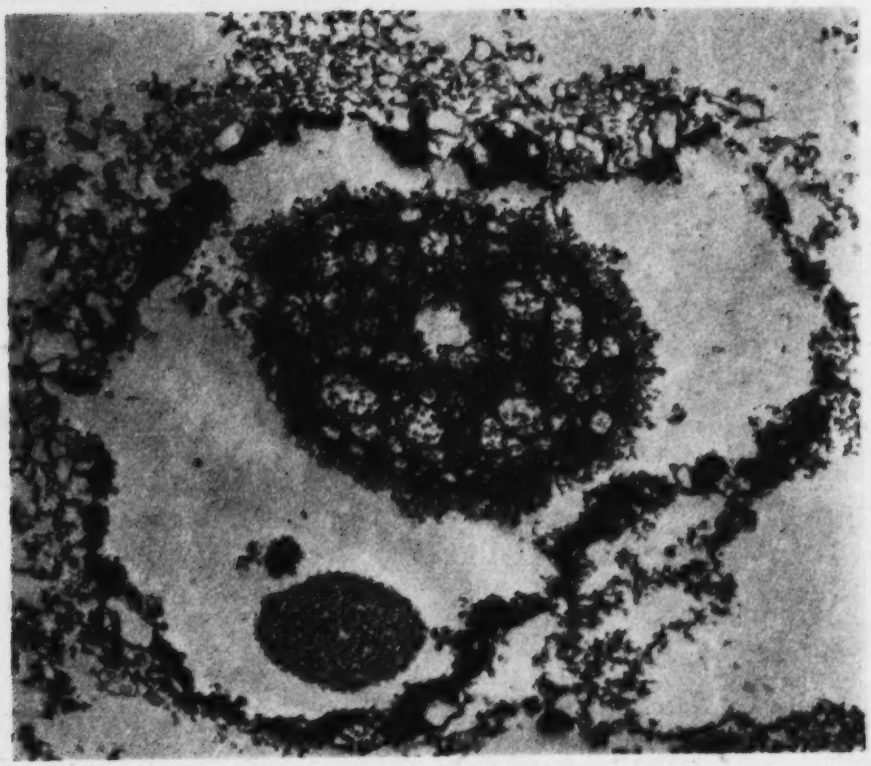

Plate 6.

Ad5-infected KB cell, 56 hours after infection, treated with 1% citric acid. Only membranous material and a few disrupted virus particles can be seen X 11,200.

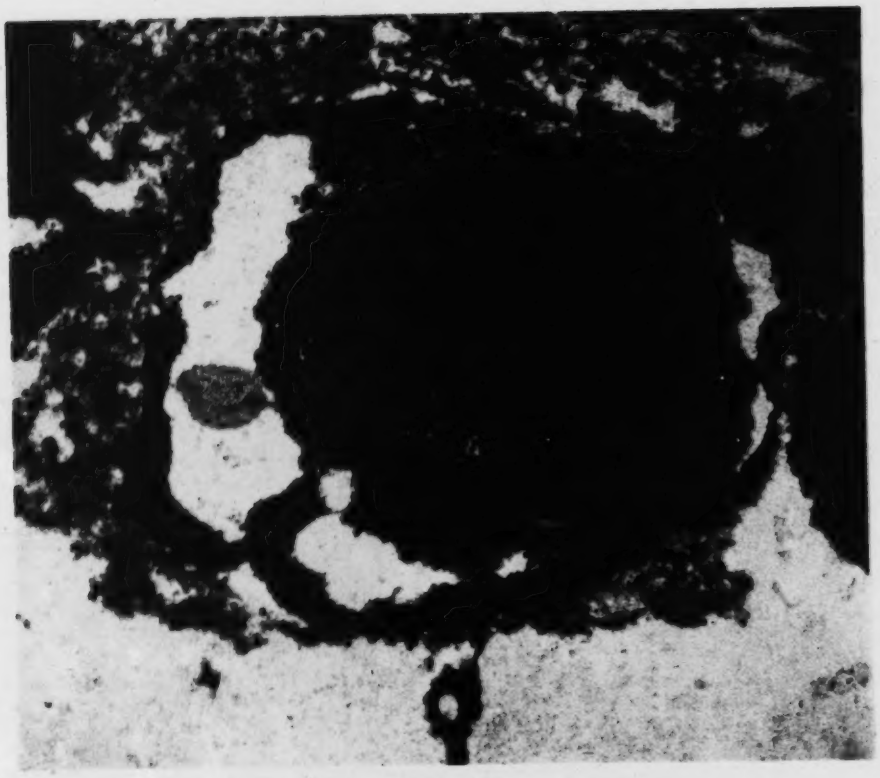

Plate 7.

Ad5-infected KB cell, 56 hours after infection, treated with 10% Tergitol. Holes in the nucleus appear corresponding to the location of the protein crystals as indicated by the presence of a partial protein crystal still seen in one of the holes. X 13,200.

with the nucleoplasm, appeared very electron dense. When uninfected KB cells were extracted with Tergitol, several cellular proteins were solubilized (see appendix for PAGE band pattern). When thin sections of these cells were observed in the electron microscope, whole nuclei which were much smaller than in the infected cells were seen. These cells revealed extensive cytoplasmic disruption, but no holes were seen in the nucleus.

d. Hypotonic buffer extraction:

Thirty ml of 0.01 M Tris-HCl buffer, 3 mM calcium chloride, pH 7.4, were added to the cell pellet. The suspension was incubated at 0-1°C with constant agitation for 15 min. The cells were then pelleted at 800 g for 10 min and embedded in Epon. Thin sections viewed in the electron microscope revealed cells at various stages of disruption (Plates 8. and 9.). The lattice structure of the crystal appeared to break down prior to solubilization and extraction of the component proteins. Of the four methods used, this seemed to cause the least amount of cellular disruption while at the same time, completely removed the crystals. Intact cytoplasmic organelles as well as intranuclear intact virus particles and adenovirus induced inclusions were still observed. When uninfected KB cells were treated in a similar

Plate 8.

Ad5-infected KB cell, 56 hours after infection, treated with hypotonic buffer for 10 min. The protein crystals have not been extracted but their fine structure has been altered. The tubular arrays are visible only in a few areas. The cytoplasm is relatively intact. X 9,500.

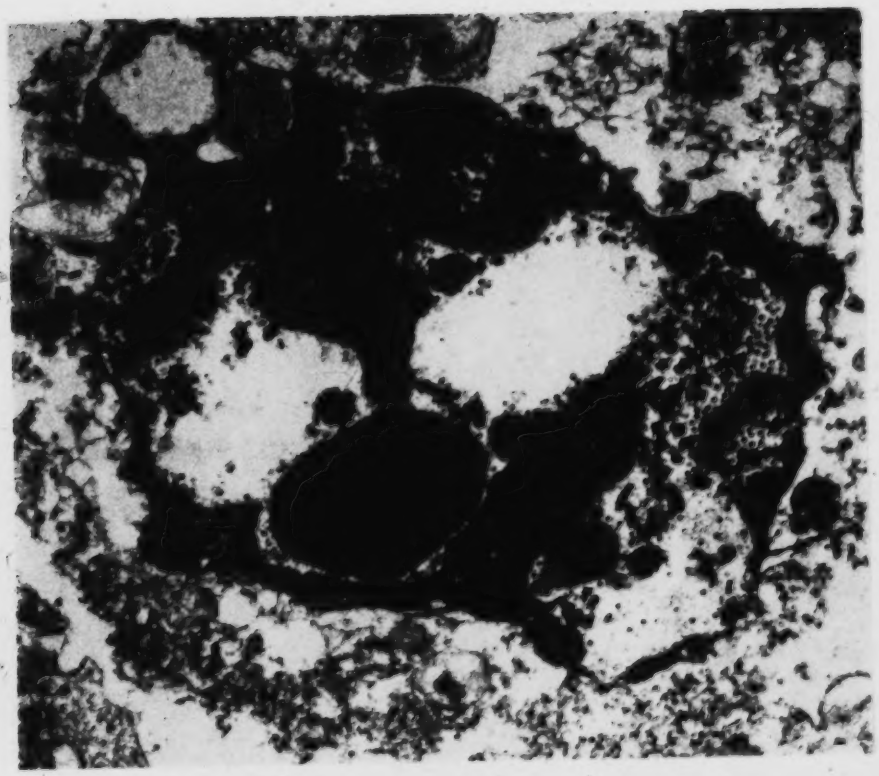

Plate 9.

A KB cell treated as in Plate 8., for 15 min. Holes in the nucleus can be seen which correspond in size and shape to the protein crystals. Other virus-induced inclusion bodies can still be seen. The cytoplasm has been disrupted, but most of the cytoplasmic remnants are still closely associated with the nucleus. X 9,500.

fashion with hypotonic buffer, very little protein was extracted as revealed by PAGE (see appendix for band pattern). The uninfected cells, when thin sectioned and observed in the electron microscope, appeared to have swollen cytoplasm, but neither membrane disruption nor nuclear extraction was apparent.

The four procedures indicate that the proteins composing the crystals are extremely soluble. When the equlibrium of the nucleoplasm (normally saturated with viral proteins) was upset, apparently by increasing the permeability of the nuclear membrane by any of the above techniques, the crystal structures quickly disappeared.

4. Extraction of Low Molecular Weight Proteins

Following centrifugation at 100,000 g for 3 hours, several batches of Tergitol-extracted proteins were subjected to acid-urea extraction in the following manner (Everitt et al., 1973): an equal volume of 10 M urea was added to the Tergitol-extracted supernatant. After 30 min at room temperature, solid sodium chloride was added to a final concentration of 2.2 M. After the salt had dissolved, 1 M citrate buffer, pH 3.1 was added to a final concentration of 0.1 M. After incubating overnight at 4°C, the extract was centrifuged at 110,000 g for 15 hours at 4°C. The cleared supernatant was dialyzed against 4 M urea in 0.1 M citrate

buffer, pH 3.4. The proteins were precipitated overnight at -20°C with 4 volumes of acetone and then lyophilized.

5. Analysis of Acid-Urea Extracted Proteins

The proteins extracted by acid-urea treatment (see 4.) from the Tergitol solution were separated by SDS-disc PAGE. Fifteen radioactive polypeptide bands were resolved (Figure 3.) When radioactively-labelled samples were analyzed electrophoretically along with labelled purified Ad5 proteins, several polypeptides seemed to have the same relative mobility as the virus polypeptides (Figure 4.) including hexon, penton base and fiber proteins.

6. Ion Exchange Chromatography

In an attempt to separate the various components of the Tergitol extracted material, ion exchange chromatography was performed in the following way.

Columns of SP-Sephadex C 50 (Pharmacia Canada Ltd. Montreal) in 0.5 M acetate buffer, 6 m urea, pH 5.3, were packed by gravity to a height of 30 cm. Samples of protein (10 mg, 93,000 cpm ^{14}C-arginine) dissolved in 0.5 ml of buffer were layered onto the column and washed with 50 ml of buffer. A linear gradient of sodium chloride (0.0 M to 0.5 M) was then applied (200 ml total volume) followed by washing with 3 M sodium chloride. Two ml fractions were

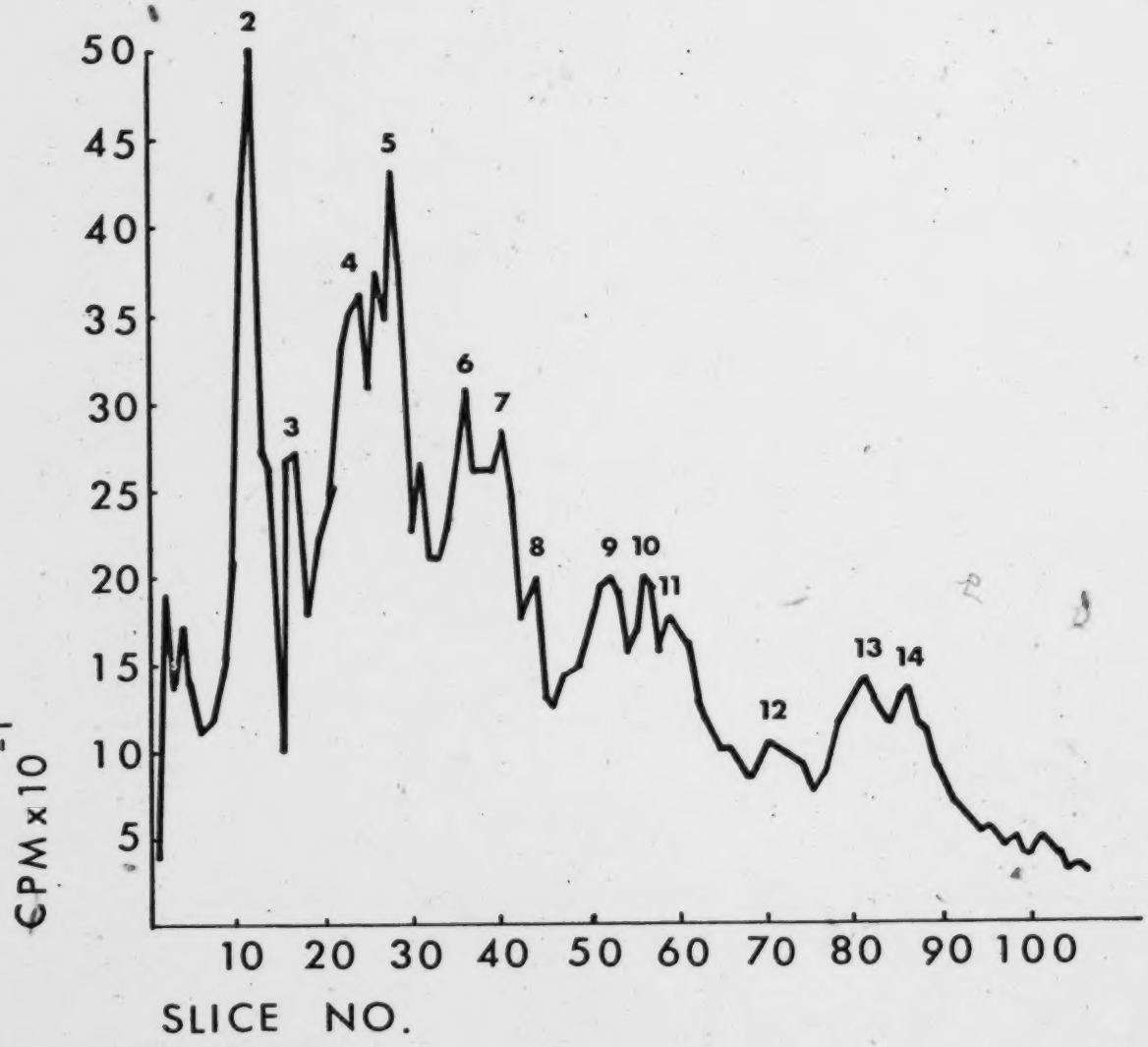

Figure 3.

Representative electropherogram of ^{14}C-polypeptides in Tergitol-exracted proteins. Proteins were extracted from infected cells labeled with ^{14}C-arginine for 10 hours at 6 hours postinfection. An appropriate amount of sample was subjected to SDS PAGE.with 3 mamps per gel applied for 3 hours. Numbers are arbitrary designations of peaks, some of which correspond to virus polypeptides. The origen (cathode) is on the left.

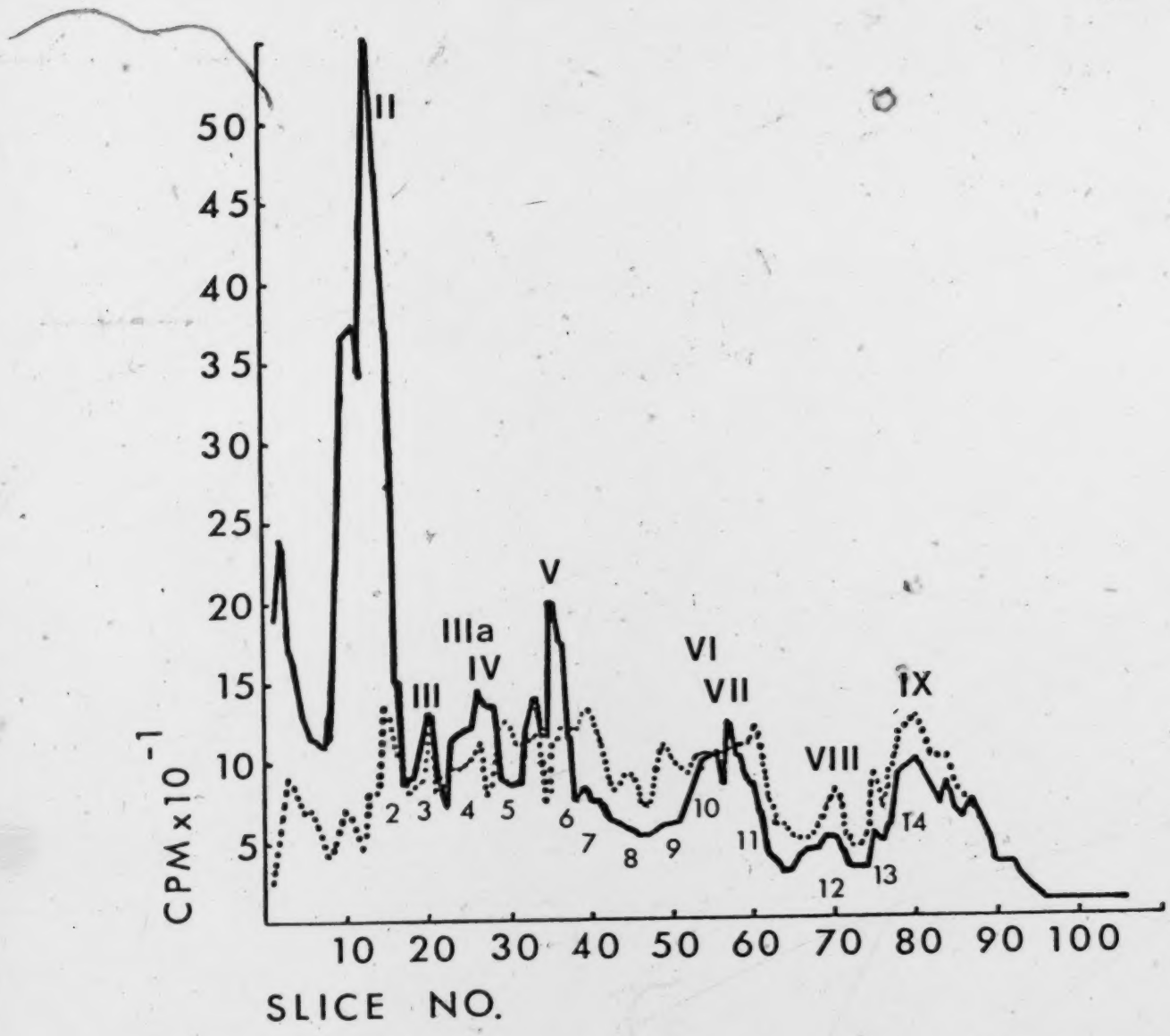

Figure 4.

Representative electropherogram of co-electrophoresis of ^{3}H-labeled purified Ad5 virions (solid line) and ^{14}C-labeled Tergitol extracted proteins (dotted line). Ad5 virions were labeled with ^{3}H-alanine while the extracted proteins were labeled with ^{14}C-arginine. Roman numerals designate viral polypeptides (see Table I), arabic numerals correspond to the peaks in Fig. 3.

collected and 0.05 ml of each were dried on filter paper and counted for radioactivity. No significant counts were obtained, suggesting that the proteins were bound too tightly to the SP-Sephadex. This matrix has a sulphopropyl functional group which is a strongly acidic cation exchanger. Therefore, similar samples were analyzed on CM-Sephadex C 50 columns. The CM-Sephadex columns have a carboxymethyl functional group which is a weakly acidic cation exchanger. A single peak, corresponding to the void volume, was obtained with this column. Similar samples were analyzed on DEAE-Sephadex A-50 columns, prepared in 0.5 M Tris-Hcl buffer, and developed with a linear sodium chloride gradient (0.0 M to 0.5 M). However, no elution of radioactive labelled proteins was observed. This type of column has been used for the separation of fiber, penton base and hexon soluble antigens (Boulanger and Puvion, 1973) The results would indicate that the proteins exposed to Tergitol may be different from the native soluble antigens, and thus could not be separated on ion exchange columns.

7. Hydroxylapatite Chromatography

Columns of hydroxylapatite (Bio-Gel HT, Bio Rad Laboratories, Richmond, California) in 0.01 M phosphate buffer, 0.1% (w/v) SDS, 1 mM dithiothreitol (DTT), pH 6.4, 0.9 cm in diameter and 20 cm high were poured over a 0.5 cm bed of Sephadex G-25. Two mg of labelled, acid-urea

extracted protein, dissolved in 1 ml of 0.01 M phosphate buffer, 1% SDS, 1% mercaptoethanol were heated to 95°C for 2 min to disrupt the secondary and tertiary protein structure and then layered onto the column. After washing the sample on with 25 ml of buffer, the column was developed with a linear gradient of 0.1 M to 0.8 M phosphate buffer containing 0.1% SDS and 1 mM DTT. Two ml fractions were collected and sampled for radioactivity. Consistent results were not obtained in different experiments as 2, 3, or 4 peaks of ^{14}C-arginine were obtained with identical samples. However, when the first two eluting peaks (always obtained) corresponding to a buffer concentration of 0.1 M and 0.18 M respectively, were analyzed by SDS-disc PAGE, they were found to contain polypeptides corresponding in mobility to virus penton and hexon polypeptides repectively (Figure 5.). The peaks were not homogenous. The last two peaks which were sometimes obtained contained fiber and lower molecular weight polypeptides.

8. Gel Filtration

Two different gel filtration systems were used: Bio-Gel A-0.5m (200-400 mesh, Bio-Rad) and Sepharose 6B (Pharmacia).

Columns, 2.4 cm in diameter and 80 cm high, were packed with Bio-Gel A-0.5m in 0.1 M phosphate buffer, 6 M urea, pH 7.3. Radioactive-labelled samples (2mg in 0.5 ml) were layered onto the column and eluted with the column buffer.

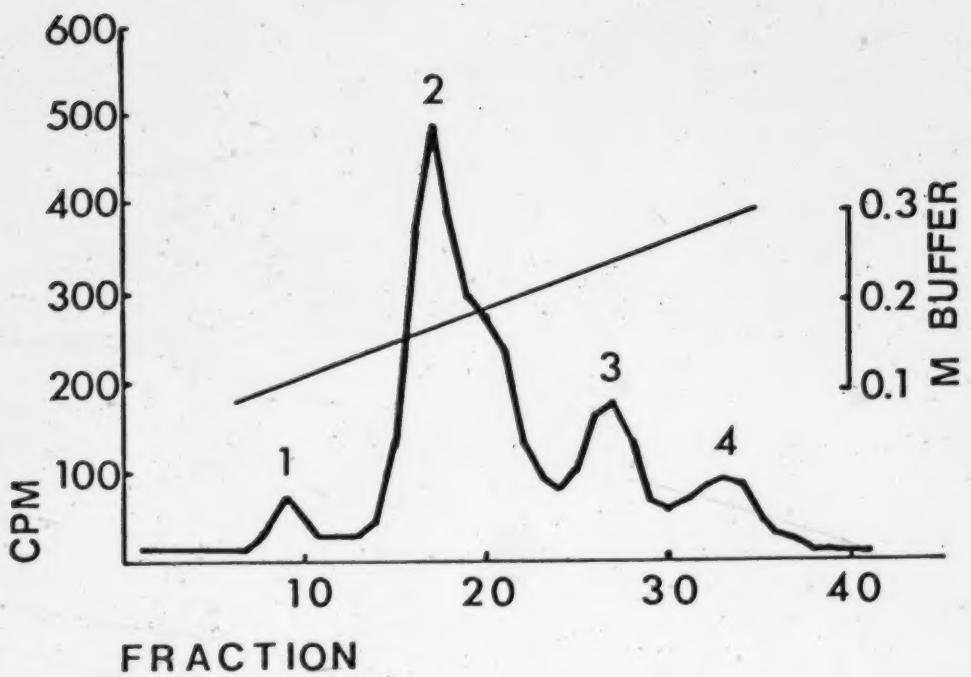

Figure 5.

Hydroxlapatite column chromatography of ^{14}C-labeled Tergitol extracted proteins in 0.01 M phosphate buffer, 0.1% SDS, 1 mM DTT, pH 6.4 eluted with a linear gradient of 0.1 M - 0.5 M phosphate buffer. (1) penton, (2) hexon, (3) fiber, (4) cores.

Fractions were collected drop-wise and sampled for radioactivity. Only one major peak, eluting in the void volume, was obtained.

Similar columns were prepared with Bio-Gel A-0.5m in 0.1 M phosphate buffer, 0.1% SDS, pH 7.4. Labelled protein samples (2 mg in 0.1 ml) were heated for 5 min in 0.1 phosphate buffer, 1% SDS, pH 7.4. After adding 0.9 ml of column buffer, the samples were layered onto the column. The column was developed with 0.1 M phosphate buffer, 0.1% SDS, pH 7.4. Fractions were collected drop-wise and sampled for radioactivity. Again, all the counts were eluted in the void volume. When this peak was analyzed by PAGE, it was found that all the proteins present in the sample were eluted in this peak, indicating that no separation of components had occurred.

In an attempt to increase the resolution of the gel filtration system, columns (2.4 cm x 80 cm) of Sepharose 6B were prepared in 0.05 M sodium choride, 0.02 M mercaptoethanol (ME), 6 M guanidinium chloride (GuHCl), pH 5.0 (Green and Bolognesi, 1974). Forty mg of protein sample were dissolved in 1 ml of 8 M GuHCl, 0.15 ml of 100% ME, 10 mg of EDTA and incubated at 50°C for 4 hours. A drop of glacial acetic acid was added to adjust the sample to pH 5-6 before layering onto the column. The column was developed with 0.05 M sodium acetate, 0.02 M ME, 6 M GuHCl. One ml fractions were collected drop-wise over a seven day period

and were subsequently sampled for radioactivity. Three major peaks of radioactivity were obtained (Figure 6.). When the fractions comprising each peak were combined, and the resulting three pools analyzed by PAGE, it was found that each peak was fairly heterogenous, containing a range of polypeptides of similar molecular weights. Peak 1 seemed to contain hexon, penton and hexon associated polypeptides ranging in molecular weight from 130,000 to 20,000. It seemed that this peak represented some type of aggregate formed when the sample was layered onto the column. Peak 2 contained polypeptides corresponding in molecular weight of 70,000 to 48,000 while peak 3 contained polypeptides ranging from 37,000 to 24,000. A small fourth peak seemed to contain polypeptides of molecular weights below 20,000. Due to the limited quantity of starting material which was available, quantities of protein recovered from these columns were very small, making further analysis impossible.

9. Hypotonic Buffer Extract Analysis

The previous experiments were conducted on only the Tergitol-extracted proteins. The following experiments and results were obtained on the hypotonic buffer extract. The supernant was centrifuged at 100,000 g in a Spinco R65 rotor, then subjected to SDS-disc PAGE. Approximately 17 polypeptide bands were resolved, 12 of which were predominant (Plate 14.). These polypeptides had identical

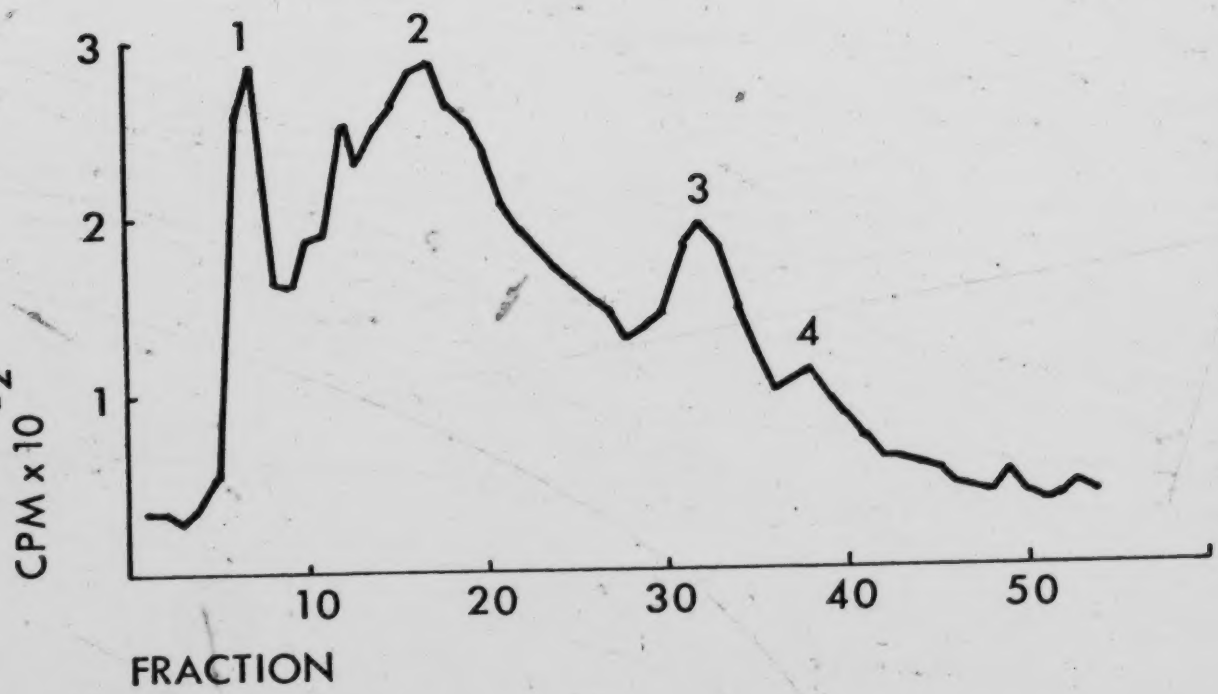

Figure 6.

Gel filtration of ^{14}C-labeled Tergitol extracted proteins in 6 M GuHCl containing mercaptoethanol at ph 5. Protein was layered on a 2.4 cm x 80 cm column of Sepharose 6B.

molecular weights to some of the polypeptides in the Tergitol extracts.

After determining its polypeptide pattern on gels, the supernatant was placed in a dialysis bag and concentrated by forced dialysis against polyethylene glycol 6000 (J. T. Baker Chemical Co.). A fine precipitate which formed was centrifuged at 100,000 g in a Spinco SW 39 rotor for 1 hour. The supernatant was removed and again analysed by SDS-disc PAGE. A similar band pattern to that found before concentrating the extract was observed. However, when the sediment was suspended in a small amount of 0.01 M Tris-HCl, pH 7.4 and analysed by SDS-disc PAGE, bands 5,7, and 8 were greatly enhanced while bands 1, 2, 3, and 4 were present in a much lower concentration than in the original extract. When the sediment was negatively stained with 1% sodium silicotungstate (SST), and examined in the electron microscope, feather-shaped, mesh-like structures as well as small, spherical bodies were seen (Plate 10.). These structures were present in such larges quantities that they completely covered the electron microscope grid. They appeared to be aggregates of one particular protein present in the crude extracts. However, these aggregates were very uniform in their morphology. When attempts were made to dilute the sample to obtain a better picture of these structures, they seemed to dissolve, indicating that they were quite soluble at lower concentrations.

Plate 10.

Appearance of the sediment after concentrating the hypotonic buffer extract with polyethylene glycol, negatively stained with 1% sodium silicotungstate (SST), pH 6.8. Feather-shaped particles are seen which closely resemble the fiber crystals reported by Maunter and Periera (1971). X 120,000.

The sediment was dialysed for 24 hours at 4°C against 1 M potasium dihydrogen phosphate (pH 4.4). When the sediment was again stained with 1% SST and examined in the electron microscope numerous crystalline arrays were seen (Plate 11). These crystalline formations consisted of bands approximately 14 nm wide, arranged in parallel rows with a periodicity of 36 nm. Each band was seen to consist of two parallel rows of beaded, electron transparent structures separated by a dark, central row. Each row of bands was connected to adjacent rows by thin filaments, running perpendicular to the bands. In some views, the bands did not show a dark central row, indicating that they might be tubular structures (Plate 12.). The crystalline arrays stained in identical ways with both 1% SST, pH 6.8 and 1% uranyl acetate, pH 4. As the in vitro crystals were very similar in morphology to metal tactoids of tropomyosin, samples of the sediment were run on analytical urea PAGE gels along with purified samples of tropomyosin. The crystal proteins had a higher molecular weight than tropomyosin, indicating that they were not tropomyosin.

When the supernatant was stored at 4°C for one month and re-examined in the electron microscope large quantities of what appeared to be hexon were seen, sometimes arranged in 2-dimensional lattices. Infrequently, six-sided aggregates of hexons, corresponding in size and shape to empty virus capsids were seen as were the small, crystalline

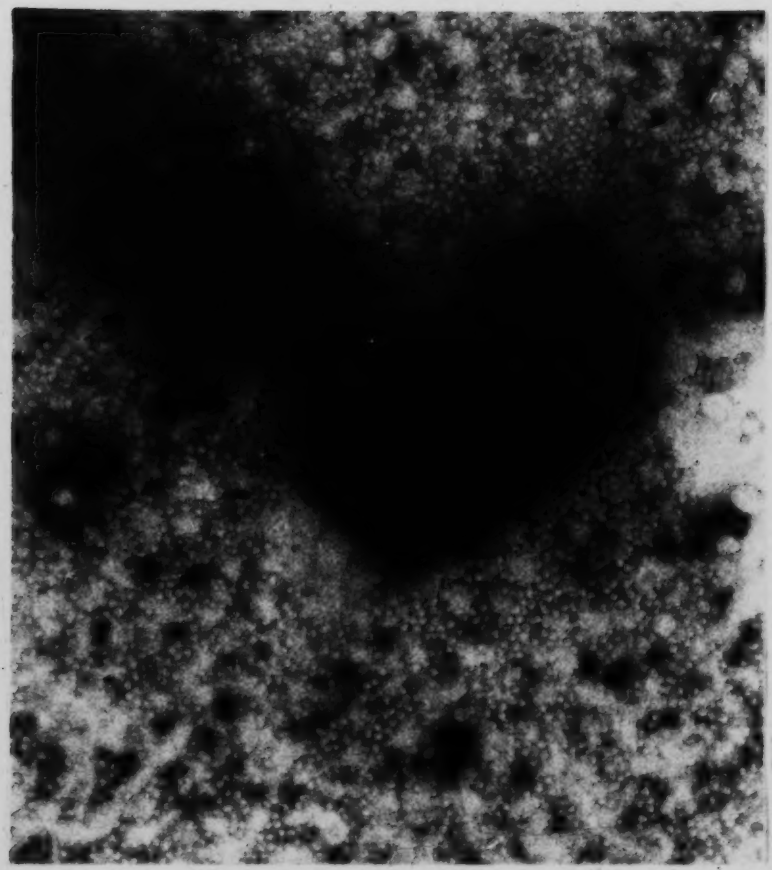

Plate 11.

Appearance of the sediment after dialysis against 1 M potassium dihydrogen phosphate, pH 4.4, negatively stained with 1% SST, pH 6.8. Beaded structures forming tubules with interconnecting fibers can be clearly seen. What appears to be hexon monomers are visible in the surrounding area. X 140,000.

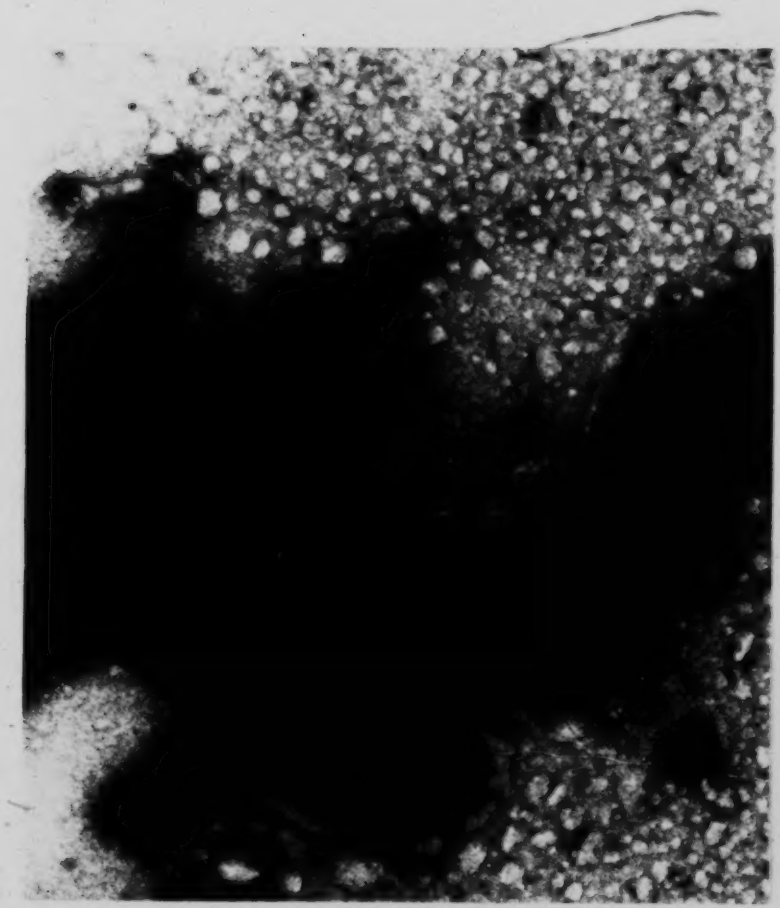

Plate 12.

Similar sample to that in Plate 11, negatively stained with 1% uranyl acetate, pH 4.8. The fine structure of the crystal can be seen. The electron transparent rows have absorbed less stain than in Plate 11. and in some areas, appear to have a central, electron transparent row. X 120,000.

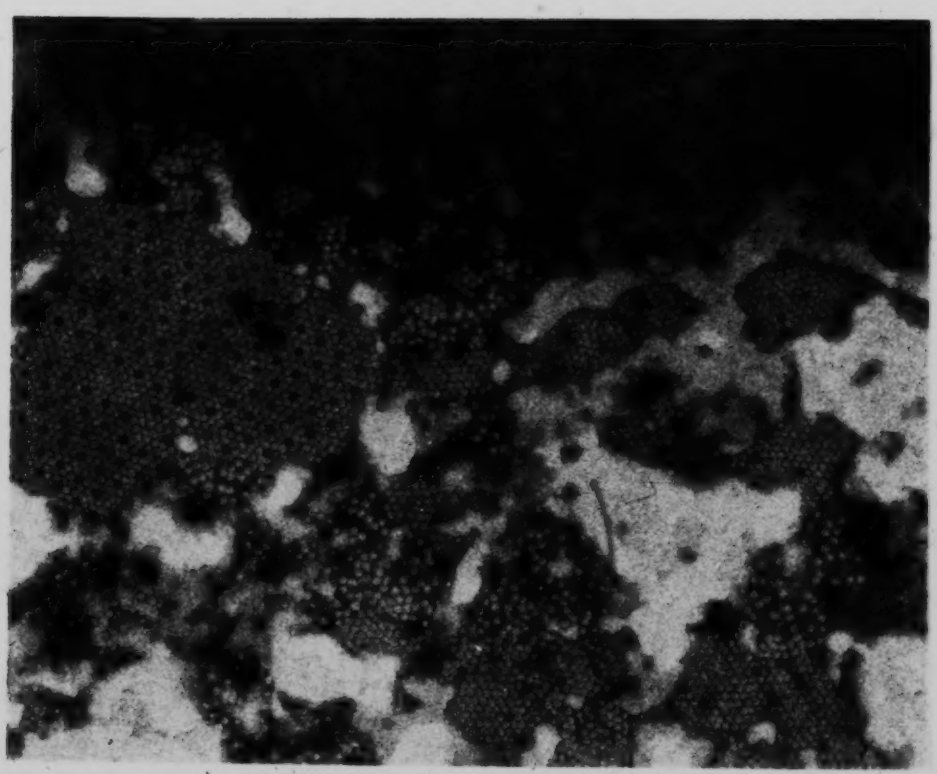

Plate 13.

Two-dimensional crystal of hexon monomers, identical to those reported recently by Pereira and Wrigley (1974). However, their hexons were derived from purified, disrupted virions while these hexons were obtained from soluble, nuclear extracts (hypotonic buffer extract). What appears to be an incomplete adenovirus shell, derived from free nonamers can also be seen. Negatively stained with 1% SST, pH 6.8. X 140,000.

arrays previously described (Plate 13.).

10. Serological Analysis

Gel diffusion plates were prepared to test the various extracts that were obtained against known antisera. Adenovirus type 5 antiserum (rabbit, GIBCO) and 'P-antigen' antiserum (kindly supplied by Dr W. Russell, London, England) were used to test the acid-soluble component of the Tergitol extract and the various components of the hypotonic buffer extract.

A positive reaction with the Ad5 antisera was obtained with both the Tergitol extracts and the hypotonic buffer extracts, resulting in several bands being visualized, corresponding to the various virus antigens present in the extracts. A weak response was observed when the extracts were tested against P-antigen antisera. This was expected as the core proteins have been shown to elicit a poor antigenic response (Prage and Pettersson, 1971).

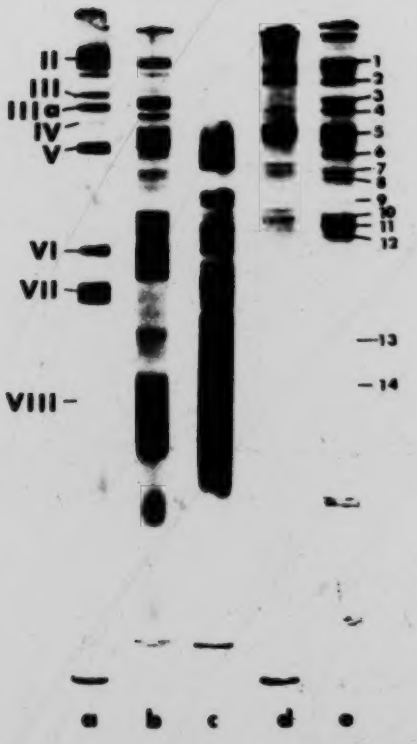

Plate 14.

SDS polyacrylamide gels (13%), stained with Coomassie Brilliant Blue R. a. disrupted purified Ad5 virions; b. acid-soluble components of disrupted purified virions; c. acid-urea treated Tergitol re-extracted with 0.2 M HCl; d. sediment from hypotonic buffer extract after concentrating with polyethylene glycol; e. supernatant from hypotonic buffer extract. Gels are from different experiments.

DISCUSSION

The results of electron microscopical and optical diffraction procedures in this study have resulted in clarification of the morphology of the intranuclear protein crystals formed during productive Ad5 infection of KB cells. By studying transverse thin sections, it was observed that the crystal consisted of an array of identical circles, arranged on an oblique lattice. The asymmetric unit of the crystal was found to be 47±3 nm in diameter with an electron-dense central ring, 22 nm in diameter, containing a central hole approximately 7 nm in diameter. The electron-dense ring appeared to consist of small ring-like subunits but these could not be resolved sufficiently in the thin sections. The electron-dense rings seemed to be connected to neighbouring rings by thin, radiating filaments. Longitudinal thin sections revealed parallel rows of double lines, corresponding in size to the electron-dense rings seen in transverse sections. These sections also revealed thin filaments between and perpendicular to the parallel rows. The rings seen in transverse sections appeared much denser than the same structures in longitudinal sections of similar thickness (approximately 65 nm). This would indicate that the electron-dense rings represent tubules or long cylindrical columns.

In transverse sections, the asymmetric units were

arranged on an oblique lattice as shown by both the optical diffraction patterns and the electron micrographs. y was designated as the unique axis, lying parallel to the long axis of the crystal and the crystal tubules. As a transverse section through the crystal would be perpendicular to this axis, it was assumed that the angle thus formed between the y axis and the xz plane was 90°. A transverse section would then represent a 2-dimensional lattice in the xz plane. By measuring the angle formed between the x and z axis on electron micrographs, and the angle formed between the x^1 and z^1 axis in reciprocal space on diffraction patterns, a beta angle of 95±2° was determined. The unit cell dimensions were obtained by measuring the inter-tubule distances on electron micrographs and were found to be: a = 50±2 nm, c = 94±3 nm, beta = 95±2°, and gamma = 90°. There were two asymmetric units per unit cell. It was concluded that the protein crystal belonged to the monoclinic system with a space group of either P2 or P2(1). By observing only transverse sections, a two-fold axis of symmetry, 2, cannot be distinguished from a two-fold screw axis of symmetry, 2(1). It should be noted that the space group P2(1) occurs very frequently in nature.

Several methods were compared for selective extraction of the protein crystals. It was apparent that at the late times after infection with Ad5 that the cells were subjected to the extraction procedures, both the cytoplasmic and

nuclear membranes were very fragile. It was also observed that whereas the Tergitol extraction procedure extracted several cellular proteins from uninfected cells, very little of the proteins were extracted when the uninfected cells were treated with hypotonic buffer. It was apparent that the uninfected cells were able to retain their integrity in this buffer and simply swell whereas the infected cells had structural changes in their membranes which allowed the viral protein crystals to be extracted. As the volume of the protein crystals in the nucleus at these late stages after infection was much greater than the small amount of cytoplasm that surrounded the nucleus, it was assumed that the majority of proteins extracted from these cells were from the virus induced protein crystals. The simplest and most efficient procedure involved suspending the cells in hypotonic buffer. All the methods were probably similar in their action - by increasing the permeability of the nuclear membrane, the protein crystals were solubilized and extracted. The crystals probably formed as a result of the saturation of the nucleoplasm with these virus-induced proteins. The crystal most probably represents the most efficient configuration for storage of these proteins. When the equilibrium of the mother liquor around the crystal was upset (increased permeability of the nuclear membrane), the crystal dissolved. At late times after infection, (when the extraction procedures were undertaken), it has been shown (Anderson et al., 1973) that the majority of polypeptides

synthesized in and extracted from infected cells were virus-induced proteins. Initial evidence for this was obtained by differential labelling infected cells and purified virus, followed by co-migration on polyacrylamide gels. This was confirmed by the positive reaction of the infected cell extracts with Ad5 antisera on immunodiffusion gels.

The failure to elute any proteins from the series of ion exchange columns used may have been the result of two factors. The smaller quantities of sample layered onto the columns in this study as opposed to the large amounts used in other investigations, may have resulted in the eluting proteins being present in quantities insufficient to detect. In addition, the detergent used for solubilization of the proteins (Tergitol) may have altered the structure of the polypeptides, resulting in their aggregation or precipitation. The lyophilized samples of Tergitol-extracted proteins were very difficult to solubilze, usually requiring heating to 95° in urea or SDS before they would dissolve. Winters and Russell (1971) have indicated that Nonidet-P40, another non-ionic detergent, affected the nuclei of adenovirus-infected cells in some way which was deleterious to assembly activity. This suggested to them that lipid or membrane-containing structures might be of importance in the assembly of the virus. It may be that these detergents affect the conformation of virus polypeptides in some way which not only affects assembly, but also their behavior on

ion exchange columns. For this reason, the hypotonic buffer method was utilized for crystal extraction. However, these extracts were not analyzed on ion exchange columns as radioactive-labelled samples were not available.

The results of the gel filtration experiments indicated that if larger quantities of proteins were available, they could be subjected to preliminary purification by exclusion chromatography (Sepharose 6B) in the presence of guanidinium chloride and mercaptoethanol. In this study, the small amounts of protein eluted from the columns were insufficient to permit for any further analysis.

The results of the hydroxylapatite chromatography indicate that there were substantial amounts of fiber, hexon and penton polypeptides present in the Tergitol extracts. This information was already known and therefore, this procedure was not further utilized.

The appearance of fiber-like crystals in negatively stained preparations as well as the relatively well defined band patterns on polyacrylamide gels of the hypotonic buffer extract indicated that it would be possible to crystallize selectively the various capsid components in the extract. The feather-shaped, mesh-like structures seen in the sediment after concentration of the extract with polyethylene glycol looked very much like the fiber crystals seen by Mautner and Pereira (1971). The same fine structure

of small double rings was apparent in some cases, although many of the "crystals" appeared to be amorphous. When this sediment was analysed by PAGE, a very predominant band, corresponding to the fiber polypeptide was seen. By lowering the pH to 4.4, crystalline arrays were formed as earlier described, but none of the fiber-like crystals were seen. Thus, by increasing the concentration of all the proteins extracted from the infected cell, and then by lowering the pH, crystallization of some of the proteins occurred. The crystalline array was easily penetrated by stain and was very susceptible to decomposition in the electron beam. This suggested that solvent played an important role in the crystal structure.

Thus, under conditions which were perhaps similar to those in the infected cell nucleus, *in vitro* crystallization of adenovirus polypeptides did occur, resulting in a structure with general similarity in size and architecture to the protein crystals seen *in vivo* .

The significance of the band pattern obtained of the hypotonic buffer extract by PAGE may be as follows. The presence of substantial amounts of hexon, penton and fiber polypeptides confirms that there is a large excess of these antigens in the infected cell nucleus, and implies that they are associated with the protein crystals. The insignificant amount of arginine-rich, major core protein (molecular weight 19,000) found in the extracts was surprising as not

only does it comprise about 20% of the total virion protein, but it has also been suggested that the crystals contained a significant amount of this protein (Marusyk et al. 1972). However, bands were seen corresponding in molecular weight to the VIa and VIb polypeptides of Ishibashi and Maizel (1974), suggesting that these precursors of the major core protein are present. Anderson et al. (1973) have shown that it takes 12 hours to convert half of the major core precursor synthesized in 1 hour to the final structural core unit. As arginine is required for assembly of virions, it may be that the conversion of these precursors to core protein (apparently a late step in assembly) is partially dependent upon external arginine. It has been suggested that in general, arginine may be involved in a transport phenomenon of viral precursor proteins to the site of assembly (Iinuma et al., 1973). This 'maturation protein' may be involved in the final stages of assembly of the adenovirus particle, that is, the formation of core protein and its integration with DNA into the virion. Thus, the protein crystals may represent the most efficient way of storing the excess structural and precursor proteins not used as substrates for virus particle formation.

Polypeptides with molecular weights of 39,000 and 35,600 (bands 10 and 11, Plate 14) were found in substantial quantities in the infected cell extracts. These may be the precursors designated Va and Vb by Ishibashi and Maizel

(1974) and may play a role in the fine structure of the protein crystal. They would be cleaved during the final virus assembly stage to the virus proteins designated VI and VIII (both of which are hexon associated, Everitt et al., 1973)

Polypeptides with molecular weights of 44,000 to 47,000 (bands 7 and 8, Plate 14) were observed both in the purified virions and the cell extracts. These polypeptides have been noted by other investigators, but their significance is unknown. They may represent precursors, intermediates or virus proteins which are present in very limited quantities. For example, the presence of an adenovirus endonuclease has been noted (Burlingham et al., 1971). Other enzymes which are responsible for the cleavage of the precursor polypeptides may also be present in low concentrations in the virus, but may accumulate in the infected cell nucleus and be detected in the nuclear extracts.

The crystals may represent a complex arrangment of hexon, fiber and core precursor polypeptides. These polypeptides may exist in a form which is not compatible with structural units of the virion. This could explain the crystallization of these polypeptides into tubular aggregates rather than their assembly. It could also represent the most efficient three dimensional arrangement of the identical protein subunits which are produced in excess and not assembled into virions, due to the relative

scarcity of a 'maturation protein'. One could speculate that hexon polypeptides may compose the central tubule with fibers radiating outwards from them. It could be that the knob end of the fiber is associated with the hexons while the end which usually comes in contact with the penton, faces outwards into the peripheral area of the crystal between the tubules. It may be that some core precursor polypeptides are associated with this distal end. This would explain why no fluoresence was seen with very specific anti-hexon antisera. The hexon antigen would be covered by the fiber core complex forming the outer surface of the crystals, and thus stain intensely with P-antigen antisera. This model is contrary to the model proposed by Boulanger et al. (1970). Their model consisted of only hexon, penton base and fiber proteins. However, they did not present any experimental evidence for this model other than stating that only structural proteins were present in their cell extracts. Due to the intense immuno-fluorescent staining of the protein crystals with P-antigen antisera, there must be some other proteins present, possibly core protein precursor components of the P-antigens. Several proteins were extracted in the present study which indicate that hexon, fiber and core precursor components were involved in the formation of the crystal. If the core precursor proteins were present in the crystals, it would seem necessary for them to be located in the peripheral area between the tubules were they would mask specific antisera reaction with

the hexon and fiber antigens, but would allow for strong reactions with the P-antigens when stained with the immunofluorescent technique. It is interesting to note that Wills et al., (1973) reported that fiber antigen was necessary for crystal formation and that the crystal structure was temperature sensitive. This could be due to the relative instability of the core precursor components at the restrictive temperatures used.

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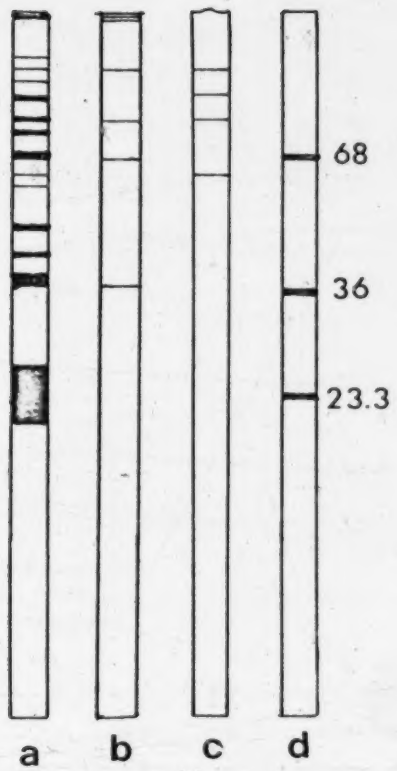
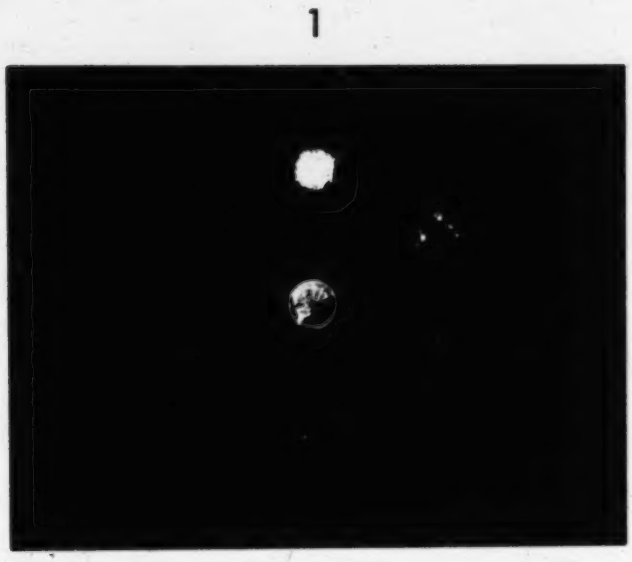
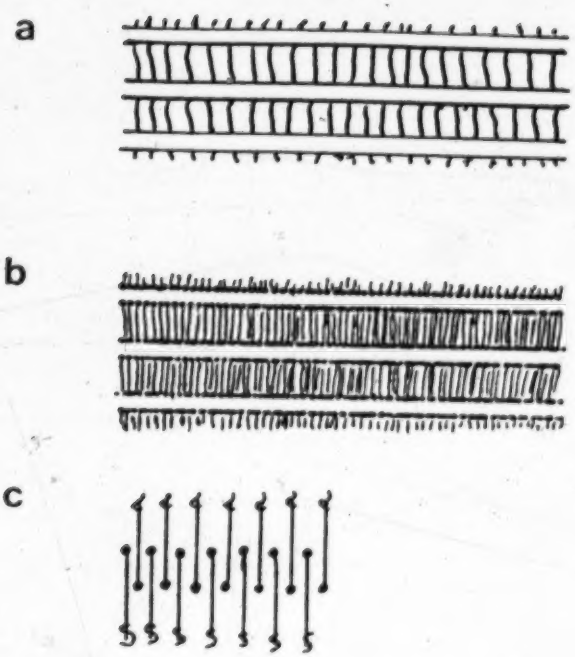
APPENDIX



Diagramatic representation of SDS-disc polyacrylamide gels. (a) representation of the band pattern obtained with the Tergitol extracts of infected KB cells, (b) represents the band pattern obtained from uninfected KB cells treated with Tergitol, (c) the band pattern obtained from uninfected cells treated with hypotonic buffer, (d) standard molecular weight markers, $\times 10^{-5}$.



Plates of gel diffusion experiments. (a) The central well contains Ad5 antisera, well 1 contained the sediment of the hypotonic buffer extract, well 3 contained purified hexon, well 4 contained the concentrated hypotonic buffer extract. (b) The central well contained P-antigen antisera, well 1 contained the sediment from the hypotonic buffer extract, well 3 contains acid souble Tergitol extract, well 4 contained the concentrated hypotonic buffer extract.



Diagrams of various crystals seen in this study. (a) represents a longitudinal section of an in vivo crystal as in Plate 2, (b) represents an in vitro crystal as in Plate 12, (c) represents a 2-dimensional model of the in vitro crystal showing presursor proteins attached to the ends of fibers. Hexon may form a complex with the fibers in the region of the tubule (central area of the model).